The role of T helper 17 cells in the Pathogenesis of Human Onchocerciasis

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Summary

Onchocerciasis, also known as river blindness, is a tropical disease causing health and socioeconomic problems in endemic communities, especially sub-Saharan Africa. The disease is elicited by a filarial parasite called *Onchocerca volvulus*, which is transmitted by the bite of infected blackflies (*Simulium).* Characteristic disease symptoms include dermatological disorders and eye lesions that can lead to blindness. Two polar forms of clinical manifestations can occur: generalized onchocerciasis (GEO), presenting mild skin disease or the hyperreactive form (HO) which exhibits severe skin disorders and inflammation. Globally, 37 million individuals are estimated to be infected. The intensity of immune reactions against the parasite is believed to influence the clinical outcomes of the disease and the immune determinants that drive tolerance or pathology are still not fully characterized. Th17 cells are a distinct subpopulation of helper T cells with potent inflammatory properties and are characterized by their predominant production of interleukin (IL)-17 (IL-17A and IL-17F). These cells are implicated in several auto-immune and inflammatory diseases. Commitment into Th17 cells or regulatory T cells (Treg) required almost the same pathway since both need TGF-β. IL-6 is one of the innate cytokines that determine the balance Th17/Treg. The present study, investigated the role of Th17 cells and the way the balance of Th17/Treg influences the outcome of clinical manifestations during human onchocerciasis. Moreover, the mechanisms underlying the plasticity between a regulatory phenotype and an inflammatory Th17 response was studied. Using a flow cytometry approach, this study investigated the expression patterns of Th1, Th2, Th17, Treg markers and other immune cells such as B cells, monocytes, CD8 and NK cells in peripheral blood mononuclear cells (PBMCs) from HO and GEO patients to putative immune/endemic normals (EN). The findings indicated that HO individuals exhibit strong Th2 and Th17 responses, as reflected by higher expression of IL-4, GATA3, and IL-17A, RORC2 expressing CD4 T cells when compared to either GEO or EN. Based on the enhanced numbers of IL-10-expressing $CD4^+$ T cells and $CD4^+CD25^+Foxp3^+$ Treg, the work also showed that patients with GEO phenotype exhibited a regulatory milieu. EN individuals on the other hand showed prominent Th1 ($CD4^+IFN\text{-}\gamma^+$ T cells). Flow cytometry data was further confirmed using a Th17 based PCR array. Th17-related genes such as IL-17 cytokine family members as well as IL-6, IL-1β and IL-22, Th17 transcription factor and signalling pathway molecules (RORC2 and STAT3) and Th2-related (IL-4, IL-13, STAT6) genes were all significantly up-regulated in HO individuals. Moreover, using *in vitro* cell cultures of PBMCs, this study further demonstrated that whereas *Onchocerca volvulus*-specific Th1 responses were increased in

cultures from EN, a strong Th2 phenotype was observed in cultures from HO individuals due to elevated levels of filarial-specific IL-5 and IL-13. To further investigate the mechanism underlying the differences between GEO and HO, an *in vitro* Th17 cell polarization model was performed and provided initial evidence that the commitment to a Th17 lineage could be modulated in PBMCs from GEO individuals by the addition of innate receptor triggering. Interestingly, it was demonstrated that products of innate stimulation, such us IL-6 and IL-1β, could also modify the GEO phenotype into one reflecting that from HO individuals. For the first time, this study provides evidence that elevated frequencies of Th2 and Th17 cells and the balance of Th17/Treg form part of the immune network instigating the development of severe pathology in human onchocerciasis. At the end a mechanism elucidating possible pathways that drive GEO and HO phenotype was proposed.

Zusammenfassung

Bei der Onchozerkose, die auch Flußblindheit genannt wird, handelt es sich um eine tropische Erkrankung, die sowohl gesundheitliche als auch sozioökonomische Probleme in den betroffenen Endemiegebieten verursacht, darunter vor allem in Afrika südlich der Sahara. Die Erkrankung wird durch die parasitische Filarie *Onchocerca volvulus* hervorgerufen, die während des Stichs einer infizierten Kriebelmücke (*Simulium*) übertragen wird. Charakteristische Symptome der Erkrankung umfassen dermatologische Beschwerden und Augenläsionen, letztere können zur Erblindung führen. Es treten zwei polare klinische Formen der Infektion auf: eine generalisierte Onchozerkose (GEO) mit einer milden Hauterkrankung oder eine hyperreaktive Form (HO), die durch heftige Hautbeschwerden und Entzündungsreaktionen gekennzeichnet ist. Weltweit sind schätzungsweise 37 Millionen Menschen infiziert. Man geht davon aus, dass die Intensität der Immunreaktionen gegenüber dem Parasiten den klinischen Verlauf der Erkrankung beeinflusst, aber die bestimmenden Faktoren, die entweder eine Toleranz oder eine Pathologie induzieren, sind noch nicht vollständig entschlüsselt. Th17 Zellen sind eine bestimmte Subpopulation der T-Helferzellen mit potentiell inflammatorischen Eigenschaften. Sie werden durch ihre vorherrschende Produktion von Interleukin (IL)-17 (IL-17A und IL-17F) charakterisiert und sind an vielen autoimmunen und inflammatorischen Erkrankungen beteiligt. Die Entstehung von Th17 und regulatorischen T-Zellen (Treg) umfasst einen ähnlichen Entwicklungsweg, da beide TGF-β benötigen. IL-6 ist eines der innaten Zytokine, welches das Gleichgewicht zwischen Th17/Treg determiniert. In der vorliegenden Doktorarbeit wurde die Rolle von Th17 Zellen während der humanen Onchozerkose untersucht und wie die Balance von Th17/Treg Zellen die klinische Erscheinungsform der Erkrankung beeinflusst. Darüber hinaus wurde der Mechanismus analysiert, dem die Plastizität zwischen dem regulatorischen Phänotyp und der inflammatorischen Th17 Antwort zugrunde liegt. In dieser Studie wurden mit Hilfe der Durchflusszytometrie die Expressionsmuster von Th1, Th2, Th17 und Treg Markern und weiterer Immunzellen, wie B-Zellen, Monozyten, CD8 und NK Zellen innerhalb der peripheren mononukleären Zellen (PBMCs) von HO und GEO Patienten untersucht und mit denen von putativ immunen/endemisch normalen (EN) Individuen verglichen. Die Ergebnisse zeigten, dass HO Patienten im Vergleich zu GEO und EN Individuen eine stärkere Th2 und Th17 Antwort besitzen, die durch eine erhöhte Expression von IL-4, GATA3, IL-17A und RORC2 exprimierenden CD4 T-Zellen gekennzeichnet ist. Diese Arbeit konnte ebenfalls demonstrieren, dass Patienten mit einem GEO Phänotyp ein regulatorisches Milieu aufweisen,

welches auf einer erhöhten Anzahl von IL-10 exprimierenden $CD4^+$ T-Zellen und $CD4^+CD25^+Foxp3^+$ Treg basiert. Im Gegensatz dazu zeigten EN Individuen eine prominente Th1 ($CD4^+IFN\text{-}\gamma^+$) T-Zellantwort. Die durchflusszytometrischen Ergebnisse wurden weiterhin mit Hilfe eines Th17 PCR Arrays bestätigt. Th17 zugehörige Gene, darunter Zytokine aus der IL-17 Familie, IL-6, IL-1β, IL-22, Th17 assoziierte Transkriptionsfaktoren und Signalwegmoleküle (RORC2 und STAT3) und Th2 zugehörige Gene (IL-4, IL-13, STAT6) waren alle signifikant in HO Individuen hoch reguliert. Des Weiteren konnte in dieser Studie mit Hilfe von *in vitro* Zellkulturen mit PBMCs dargestellt werden, dass *Onchocerca volvulus*-spezifische Th1 Antworten in Kulturen von EN erhöht waren, wohingegen ein starker Th2 Phänotyp in Kulturen der HO Individuen vorlag, der durch gesteigerte filarienspezifische IL-5 und IL-13 Spiegel deutlich wurde. Um weiterhin den Mechanismus zu untersuchen, der zu den Unterschieden zwischen GEO und HO Individuen führt, wurden *in vitro* Th17 Polarisierungsexperimente durchgeführt. Diese erbrachten den Nachweis, dass eine Polarisierung in Richtung der Th17 Linie in PBMCs aus GEO Individuen durch die Stimulation innater Rezeptoren moduliert werden kann. Interessanterweise konnte gezeigt werden, dass Produkte der innaten Stimulation wie z.B. IL-6 und IL-1β ebenfalls den GEO Phänotyp so verändern konnten, dass er demjenigen der HO Individuen entsprach. Zum ersten Mal konnte diese Arbeit aufzeigen, dass eine erhöhte Frequenz von Th2 und Th17 Zellen sowie das Gleichgewicht zwischen Th17/Treg Zellen einen Teil des immunologischen Netzwerkes bilden, der die Entwicklung von schweren Pathologien initiiert. Anhand dieser Ergebnisse wurde ein möglicher Mechanismus skizziert, der die Entstehung der unterschiedlichen Phänotypen von GEO und HO Individuen erklärt.

THIS THESIS IS BASED ON THE FOLLOWING ORIGINAL PUBLICATIONS

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Oral presentation, Mixed Th2/Th17 immune responses are associated with immunopathology in human onchocerciasis at the 18th Symposium "Infection and Immune defence".12- 14.03.2013, Burg Rothenfels, Germany.

Poster presentation, Hyperreactive Onchocerciasis is Characterized by a Combination of Th17-Th2 Immune Responses and Reduced Regulatory T cells at the 44th Annual Meeting German Society for Immunology (DGfI) September 17–20, 2014 • Bonn/ Germany.

Poster presentation, Hyperreactive Onchocerciasis is Characterized by a Combination of Th17-Th2 Immune Responses and Reduced Regulatory T cells at the Cluster Science Days 2014 – November 3-4, 2014

List of abbreviations

AAM	Alternatively activated macrophages
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Allophycocyanin
B reg	Regulatory B cells
CAM	Classically activated macrophages
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acids
CTLA-4	Cytotoxic T lymphocyte antigen 4
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EN	Endemic normal
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein3
GATA3	GATA binding protein 3
GEO	Generalized onchocerciasis
GITR	Glucocorticoid induced tumor necrosis factor receptor
GITRL	Glucocorticoid induced tumor necrosis factor receptor ligand
HO	Hyperreactive onchocerciasis
IFN-Ɣ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin gamma
IL	Interleukin
L3	Filarial larvae stage 3
mAb	Monoclonal antibodies
MACS	Magnetic-activated cell sorting
MDP	Muramyl dipeptide
MF	Microfilariae
mRNA	Messenger ribonucleic acid
NaCl	Sodium Chloride
NK	Natural Killer cells
NKT	Natural Killer T cells
NLR	Nod-like receptor

O.v.	*Onchocerca volvulus*
OvAg	*Onchocerca volvulus* antigen
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine 5
PerCP-Cy5.5	Peridinin chlorphyll protein-cyanine 5.5
PGN	Peptidoglycan
ODN	Oligodesoxynucleotide
LPS	Lipopolysaccharide
HKLM	Heat killed *Listeria monocytogenes*
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
RORC2	retinoic acid receptor-related orphan receptor C2
Rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
STAT3	Signal transducer and activator of transcription 3
STAT6	Signal transducer and activator of transcription 6
T bet	T-box transcription factor
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cells
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Tr1	Regulatory type 1 cells
Treg/Tregs	Regulatory T cells
Vs	Versus
αCD3/αCD28	anti-CD3/anti-CD28

Table of Contents

1. Introduction

Filariae are a distinct group of parasitic nematodes that can infect all classes of vertebrates other than fish [1-3]. The name filarial is derived from the Latin word *filum* meaning thread and refers to the thread-like morphology of the worms [2, 3]. They share similar features, including an adult stage that resides outside of the digestive tract, the requirement of an obligatory intermediate host (hematophagus arthropod) and the release of microfilariae into the definitive host [3, 4]. Eight filariae elicit lymphatic and tissue filariasis in human. Over 140 million people are infected with *Wuchereria bancrofti, Brugia malayi, B. timori* that elicit lymphatic filariasis. *Onchocerca volvulus, Loa loa, Mansonella perstans, M. streptocerca* and *M. ozzardi* are responsible for eliciting tissue filariasis. According to recent estimation, more than 200 million individuals are infected with lymphatic filariasis and onchocerciasis. A common characteristic of infections with filarial nematodes is that they enter the host silently and persist many years by manipulating host responses and inducing evasion strategies to avoid overt responses by the host's immune system [5-8]. In the majority of infected individuals, there is a striking absence of disease. However, there are several million infected people who develop pathological forms of disease that have grave socioeconomic consequences [7, 9-11]. The disability-adjusted life years (DALYs) are estimated to be in the region of 6.3 million years for lymphatic filariasis and onchocerciasis [12-16]. All filariae that can infect humans are classified into two main groups: filarioidae or Onchocercidae [17]. This thesis focuses on *Onchocerca volvulus,* a nematode which belongs to the family of Onchocercidae [17].

1.1. Human onchocerciasis

The genus Onchocerca contains several species such as *O. ochengi*, *O. dukei, O. gibsoni* which infect cattle and *O. volvulus* which is responsible for onchocerciasis in man[18]. Onchocerciasis, commonly known as river blindness, is a filarial infection of the eyes and skin and is transmitted through bites of *Onchocerca volvulus*-infected black flies (*Simulium spp*) [19]. This section provides an overview about our current understanding of parasite distribution and epidemiology, life-cycle, clinical manifestations, diagnostics and treatment of the disease.

1.1.1. Distribution

In 2012, approximately 130 million people were at risk of infection in 27 African countries [20]. The 3 countries with the largest "at risk" population are Nigeria (37 million), the Democratic Republic of the Congo (30 million) and Ethiopia (8 million) [20, 21]. Although 99% of infected people live in Africa, small pockets of endemicity can be found in Yemen and Central and Southern America [22]. Over 37 million people are currently infected and within those, around 1% develop hyperreactive forms of the disease [4, 7]. Figure 1.1 depicts the global distribution of the disease.

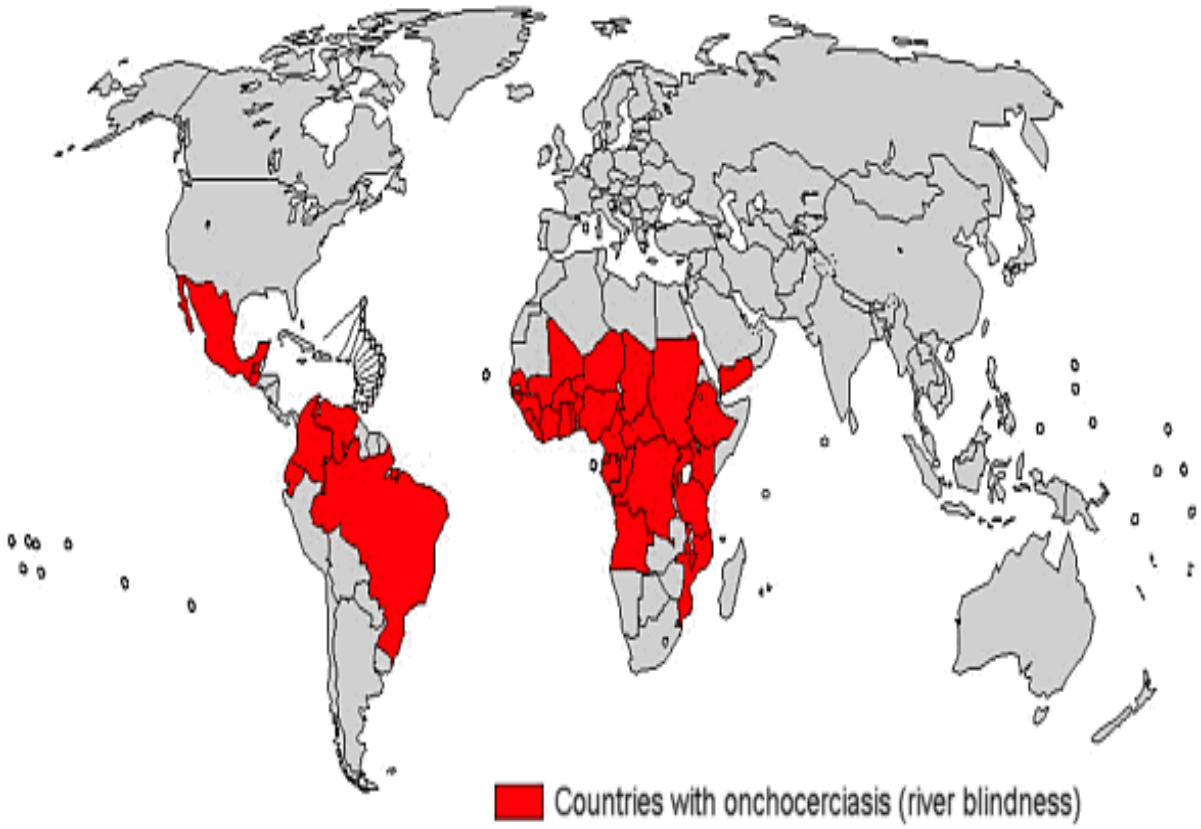

Figure 1.1: Geographical distribution of human onchocerciasis.

The distribution of onchocerciasis is linked to the location of blackflies which are naturally found close to fast-running streams and rivers in the inter-tropical zones(red). About 90% of disease cases occurs in Africa. Onchocerciasis is also found in six countries in Latin America and in Yemen in the Arabian Peninsula. Image obtained from http://www.who.int/blindness/partnerships/onchocerciasis_disease_information/en/

1.1.2. *Onchocerca volvulus* life cycle

The life cycle begins when a parasitized female black fly of the genus *Simulium* takes a blood meal. During this blood meal, third-stage filarial larvae are released onto the skin of the human host, where they penetrate into the bite wound [7]. The larvae then develop into adult filariae in subcutaneous tissues and commonly reside within nodules in the subcutaneous connective tissues. Female adult worms can live in these nodules for approximately 15 years [4]. Male worms visit the nodules for mating. Adult females release approximately 1000 to 3000

microfilariae per day over a 9 to 14 year period [9, 10, 22, 23]. The cycle is completed when microfilariae are taken up during a further blood meal [7, 23]. After ingestion, the microfilariae migrate from the blackfly's midgut through the hemocoel to the thoracic muscles. The microfilariae then develop into first-stage larvae (L1) which mature over a course of 10 to 12 days to infective stage three larvae (L3) [7]; thereby completing the life cycle [24-26]. Figure 1.2 illustrates the complete life-cycle of *Onchocerca volvulus*.

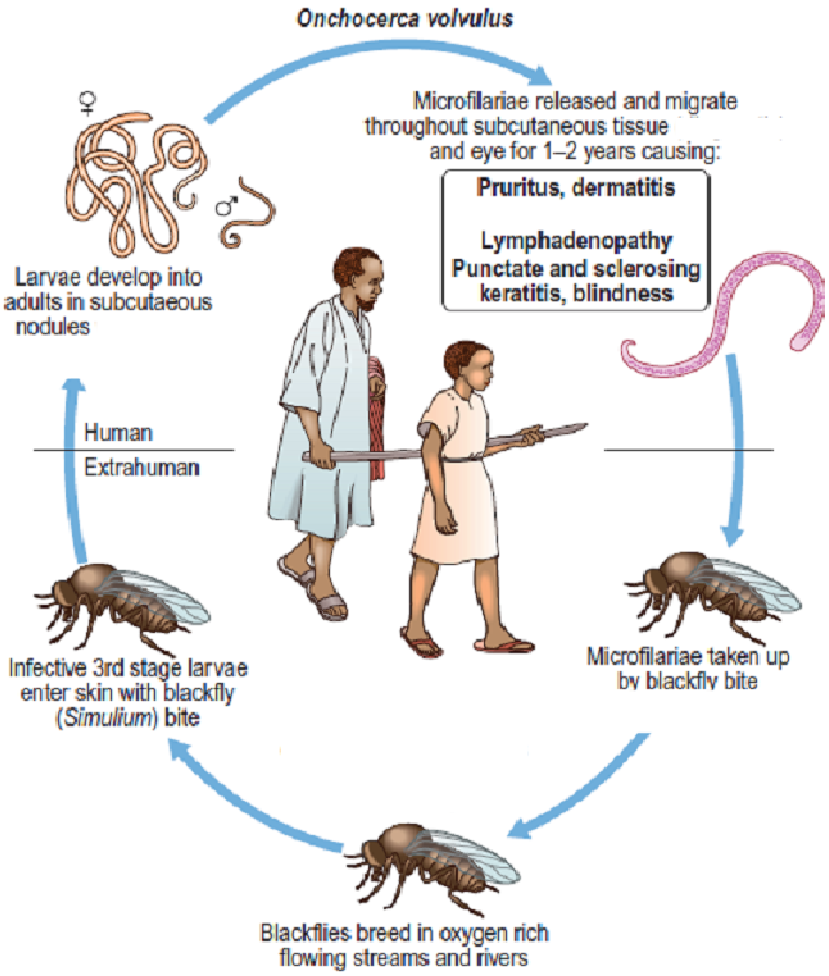

Figure 1.2 Life-cycle of *Onchocerca volvulus*

Adult filarial worms release millions of microfilariae into the skin which are taken up by blood feeding *Simulium* blackfly vectors. Within the vector's midgut, the parasite metamorphosis's into L1 and then L3 larvae. The latter are released into the human host during a further blood meal. Image from [26].

1.1.3. Clinical manifestations

The pathogenesis of filarial infection is characterized by acute and chronic inflammation [27-29]. Disease manifestation is believed to be provoked by the parasite, the host's own immune reaction and/or consequent additional infections. In human onchocerciasis, the clinical manifestations are thought to be associated with the destruction of the microfilariae in the skin and eyes [4, 30, 31]. The symptoms of disease manifestations are mainly keratitis, chorioretinitis and various forms of dermatitis [9, 23]. Infection characteristics have divided exposed and infected individuals into three groups: Generalised Onchocerciasis (GE0), Hyperreactive Onchocerciasis (HO) and putative immune/Endemic normals (EN) [32-34]. The GEO group encompasses a spectrum of clinical manifestations and host immune responsiveness and individuals present high parasite loads but mild pathology. Hyperreactive Onchocerciasis (HO) individuals on the other hand, have few worms but severe pathology [4, 6, 10, 35]. In endemic areas, putative immune (PI) individuals or endemic normals (EN) are persons, who despite permanent exposure to the parasite, remain without infection or clinical signs of disease [6, 35].

1.1.3.1. Dermal pathology in onchocerciasis

The principal site of infection is the skin which contains masses of microfilariae [4, 36, 37]. Dying microfilariae can elicit a subcutaneous inflammatory response, a condition often called “lizard skin". Over time the skin may lose its pigmentation, which on dark skin gives rise to an appearance commonly referred to as “leopard skin”. As mentioned above the majority of infected people develop the generalized form the disease, characterized by high worm and mf loads with mild skin inflammation. This is in contrast to the small group of individuals who develop a

localized or HO form of disease also known as sowda (Arabic word for black) which refers to disease manifestations only on one side of the body [4, 10, 35].

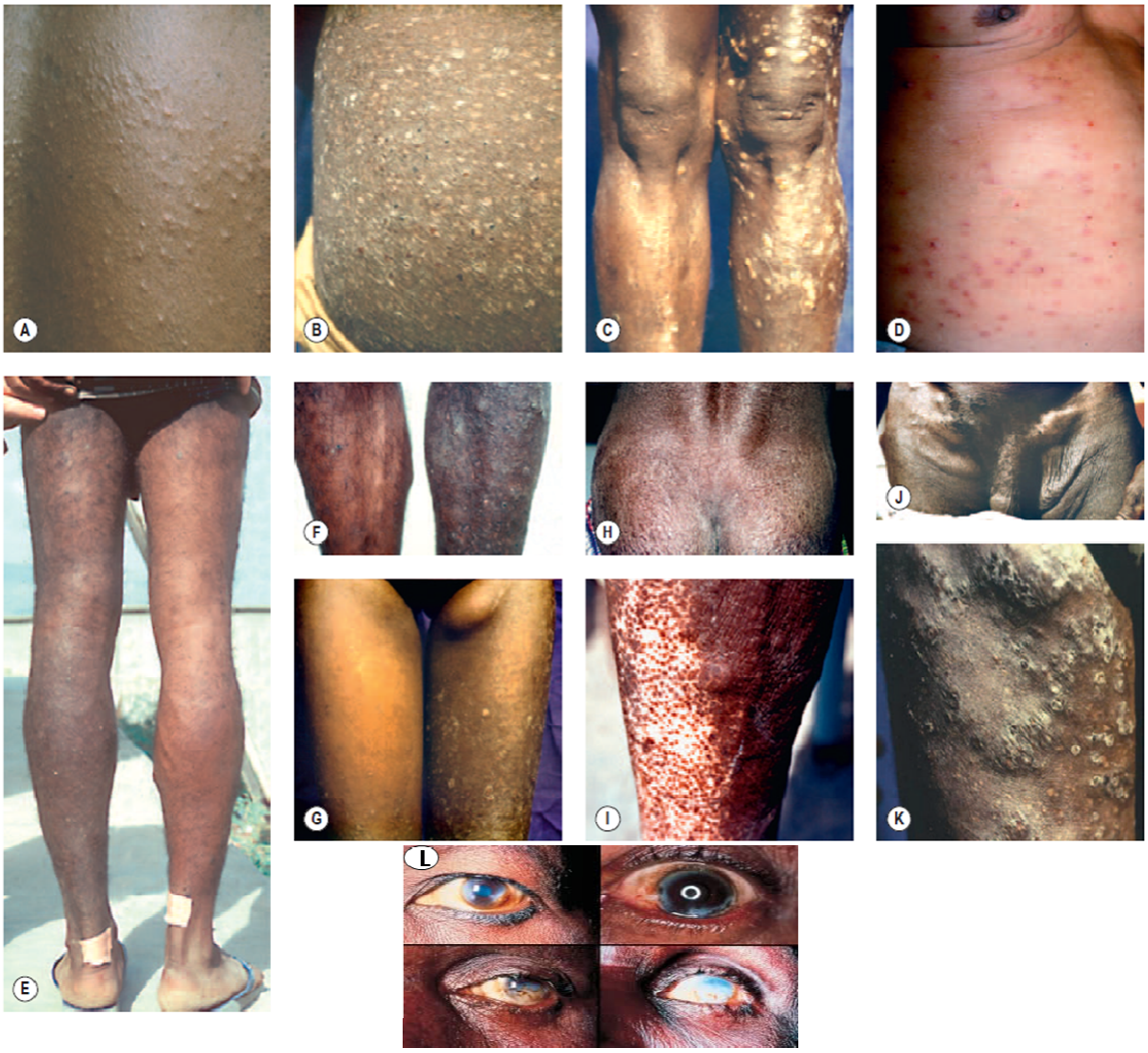

Figure 1.3: Forms of diseases associated with human onchocerciasis.

(A) Acute pustula onchodermatitis. (B, C) Chronic onchodermatitis. (D)= Dermatitis in Guatemala, erisipela de la costa. (E,F,G) Sowda with unilateral preference and dark skin as well as swollen femoral lymph node. (H) skin atrophy in 15-years old boy. (I) Leopard skin. (J) Hanging groin. (K) Lichenifield onchodermatitis. (L) Ocular onchocerciasis. Image adapted from [26] and http://www.cehjournal.org/article/vision-2020-update-on-onchocerciasis.

The sowda form was first described in Yemen [38] but has since been reported in West Africa [39] and eastern Sudan [40]. Murdoch *et al* classified and graded the cutaneous changes in acute papular, chronic papular and lichenified or reactive onchodermatitis, atrophy and depigmentation (leopard skin) [37, 41]. Figure 1.3 presents examples of skin lesions in onchocerciasis. Many patients develop

fibrous subcutaneous masses containing the adults worms termed onchocercomata (nodule) [6, 42]. The nodules vary in size from 1 to 5cm in diameter. The numbers of nodules vary and an infected individual may have as many as several hundred [42-44].

1.1.3.2. Ocular onchocerciasis

Worldwide, onchocerciasis is the second leading cause of blindness stemming from an infectious origin. Microfilariae that migrate into the eye, and subsequently die there, can cause severe inflammation and scarring that can lead to impaired vision and blindness [23, 45, 46]. The severity of ocular pathology in distinct regions of West Africa is due in part to infection with different strains of the parasites [47, 48]. The life expectancy of a person who becomes blind in endemic areas may be shortened by about 10 years [23, 46, 48]. Onchocerciasis is also referred to as “river blindness” because of the blindness it causes and its association with habitation near rivers where blackflies breed [49, 50]. An illustration of ocular onchocerciasis is shown in figure 1.3.

1.1.4. Diagnosis

A physical examination or palpation of the patient can detect localized dermatitis or subcutaneous nodules. Usually, the presence of microfilariae is diagnosed by a biopsy of the skin called a skin snip. Microfilariae are spontaneously released from the skin when the skin snip is placed in a saline solution [43, 51, 52]. Subcutaneous nodules can also be surgically removed to identify the presence of adult worms [43, 44]. Polymerase chain reaction (qPCR) is a good approach used to diagnosis early infected people but such techniques are often impractical and expensive for local health care centres [53]. Immunodiagnostic tests such us

standard ELISA, dot blot ELISA and Western blot have also been developed to test for infection [54-56].

1.1.5. Treatment

The standard microfilaricidal drugs ivermectin and albendazole are used in mass drug administration programmes, with the aim of interrupting transmission. These programmes aim at reducing the burden of infection and, in some situations, leading to regional elimination of onchocerciasis [21, 57]. The recommended treatment for onchocerciasis is ivermectin (Mectizan®) [16, 21, 57, 58], which targets microfilariae and is fast-acting, safe, and effective. Ivermectin kills circulating microfilariae as well as those that are still in adult female worms; this reduces the numbers of microfilariae in the skin and the production of new microfilariae by adult worms so the disease does not progress [57, 58]. Standard treatment is once a year, but infected people can be treated up to three times in one year. Since adult worms are not killed and will continue to reproduce, treatment must continue for 10 to 15 years [21, 57, 59]. Before any treatment for onchocerciasis is begun, it is imperative to ascertain which individuals or communities are heavily infected with *Loa loa*, another filarial disease [57]. Ivermectin can provoke severe side effects, even death in patients with *Loa loa* [57]. In terms of macrofilaricidal drugs, the only safe option is the administration of antibiotics such us tetracyclines and doxycycline [60-65]. This therapy targets *Wolbachia,* the essential endosymbiont bacteria within the majority of filariae pathogenic to man. *Wolbachia* reside within the embryos of female worms and are required for their reproductive processes. Elimination of *Wolbachia* causes MF death and eventually destroys the worms as well [63, 66-69].

1.2. Immunity in human onchocerciasis

The host immune response towards onchocerciasis involves a range of innate and adaptive pathways [70-72]. Previous studies have noted a strong regulatory phenotype in generalized onchocerciasis individuals (high levels of IL-10, TGF-β, regulatory T cells and IgG4) and this immune balance is thought to be induced by the filariae to avoid effector immune responses [5-7, 73]. In contrast, HO patients display elevated IgE, IL-4 and severe symptoms of pathology. This section provides a brief synopsis about cell subtypes involved in human onchocerciasis.

1.2.1. Macrophages and monocytes

Monocytes circulate in the blood and are the precursors of macrophages [74, 75]. Macrophages are a heterogeneous population of phagocytic cells that reside in most tissues of the body [76]. In general, there are two subpopulations of activated macrophages: The classically activated macrophages (M1 or CAM) which are activated through inflammatory signals such as IFN-γ and the alternatively activated macrophages (M2 or AAM) which are provoked through anti-inflammatory conditions such as Th2 signals like IL-4 [77]. Helminth infections are known to induce a Th2 responses that polarize macrophages towards an M2 phenotype [78]. In onchocerciasis, M2 macrophages appear to suppress the immune response against the parasites, promoting anergy and/or tolerance [79]. It has been hypothesized that M2 macrophages may facilitate the repair of tissues that have been mechanically damaged by helminths [80].

1.2.2. Granulocytes

Granulocytes comprise of a granulated cytoplasm containing granules rich in immunomodulatory molecules. They can be subdivided into neutrophils, eosinophils, basophils and mast cells. Granulocytes play a key role in innate and

adaptive immunity and can secrete a wide variety of mediators without *de novo* protein synthesis [81]. The capability of granulocytes to release toxic cationic proteins is an effector mechanism against extracellular organisms such as helminths [81, 82], although these molecules have also been implicated in tissue damage [83]. Thus, granulocyte-mediated immunopathology has been observed in hyperreactive onchocerciasis individuals [42]. In helminth infections, ligation of parasite-specific immunoglobulins to Fc receptors initiates antibody-dependent cytotoxicity (ADCC) killing of the parasites [84]. The release of granule proteins can be induced through binding of antigen-IgE complexes to the high affinity IgE receptor (FcεRI) that triggers a tightly controlled phosphorylation cascade [85, 86]. Higher eosinophil numbers and their related factors (e.g. eosinophil cationic protein) and neutrophils were shown to be associated with MF individuals [51]. Further studies have implicated eosinophils in *Onchocerca volvulus*-induced atopic dermatitis and keratitis which was indicated by the release of granules such us Eosinophils-granule major protein [87, 88].

1.2.3. Natural Killer and Natural Killer T Cells

Natural killer (NK) cells are innate lymphocytes that produce important amounts of immunomodulatory cytokines after their receptor engagement by glycolipid antigens presented in CD1 molecules [89]. Thus, NK cells play an important role during infection and mainly against intracellular microorganisms [90]. Different NK cell subsets have been determined according to the presence and expression intensity of CD3, CD16 and CD56 surface molecules [91]. Mostly, NK cells are referred as NKT cells or NK cells according to their expression or not of the T cell receptor molecule (CD3) respectively [91, 92]. Studies have addressed the potential for NK cells to either contribute to, or to modulate, immune responses to

helminths [92]. Antibody opsonisation of parasites can facilitate the activation of NK cells via the ligation of FcγRIII which lead to ADCC and lysis of the opsonised parasites [85, 92]. *Babu et al.* demonstrated through *in vitro* cultures of PBMCs that NK cells can be activated by filarial parasites and subsequent cell death [93]. Reduced NK and NK T cell numbers have been observed in generalized onchocerciasis individuals [94].

1.2.4. B cells and Immunoglobulins

B cells are a heterogeneous population of lymphocytes that can react to antigens. This antigen recognition can be in a T-dependant or T-independent manner [95, 96] . In addition to antibody production, B cells perform other substantial roles such as antigen-presentation, co-stimulatory molecule signalling, and cytokine production. Nevertheless, the importance of B cells in driving a T dependent response can vary with the particular antigen and the type of immune milieu [97, 98]. Humoral immunity has been shown to be involved in protective immunity in helminth infection. For instance, ADCC is an important mechanism by which parasites are killed [85]. Hence, during helminth infection a polarized Th2 response induces B cells to produce IgE and IgG1 [6, 99]. These antibodies induce degranulation in eosinophils and other granulocytes and can activate the complement system which leads to the clearance of the infection [82, 85, 100, 101]. In *O. volvulus*-infected individuals , the induction of IgG4 is associated with the regulatory phenotype in GEO patients [6, 73, 101, 102].

1.2.5. CD4 helper T cells

CD4 helper T cells differentiate into various subsets of effector T cells that have a variety of functions. There are four main functional classes: Th1, Th2, Th17 and the regulatory T cells. They are distinguished principally by the release of specific

cytokines and up-regulation of unique transcription factors. Th1 cells are characterized by the secretion of IFN-γ and the transcription factor T bet; Th2 by IL-4, IL-5, IL-13 and GATA3; Th17 by IL-17A and RORC2 and regulatory T cells secrete IL-10 and/or TGF-β and express CD25 and Foxp3 [103, 104]. $CD4^+$ T cells play an important role in the protection, susceptibility and modulation of human onchocerciasis. T cell immune responses to *O. volvulus* are very distinct in generalized, hyperreactive onchocerciasis and endemic normals [7, 10, 105]. For example, Th1 responses were observed to be associated with endemic normals whereas both Th1 and Th2 were linked to hyperreactive onchocerciasis [94, 106-109]. In addition, individuals without skin lesions, have pronounced systemic Th2-type responses with a trend of increasing intensity in the order of depigmentation, papular dermatitis, atrophy and lichenified dermatitis [110]. Immunohistology of ectopic secondary lymph follicles in subcutaneous nodules from patients with hyperreactive onchocerciasis showed the presence of $CD3^+CD45RO^+$ T cells in the mantle zone [42]. Immunosuppression in chronic onchocerciasis has been observed to be associated with antigen-specific Tr-1 cells [111] and also higher regulatory responses characterized by the presence of $CD4^+CD25^+Foxp3^+$ T cells were shown in generalized onchocerciasis [6, 10, 112, 113]. *Mitre el al*. demonstrated that IL-10-producing $CD4^+$ T cells were predominant in filarial-infected patients [114]. The implication of Th17 responses in onchocerciasis has not yet been established but they have been shown to be involved in the pathogenesis of lymphatic filariasis and schistosomiasis [115, 116]. As mentioned above, GEO is associated with a strong regulatory phenotype and research predicts that these characteristic are induced by the helminth to avoid host effector

immune responses [5-7, 73]. Figure 1.4 presents the potential immune networks that arise during human onchocerciasis.

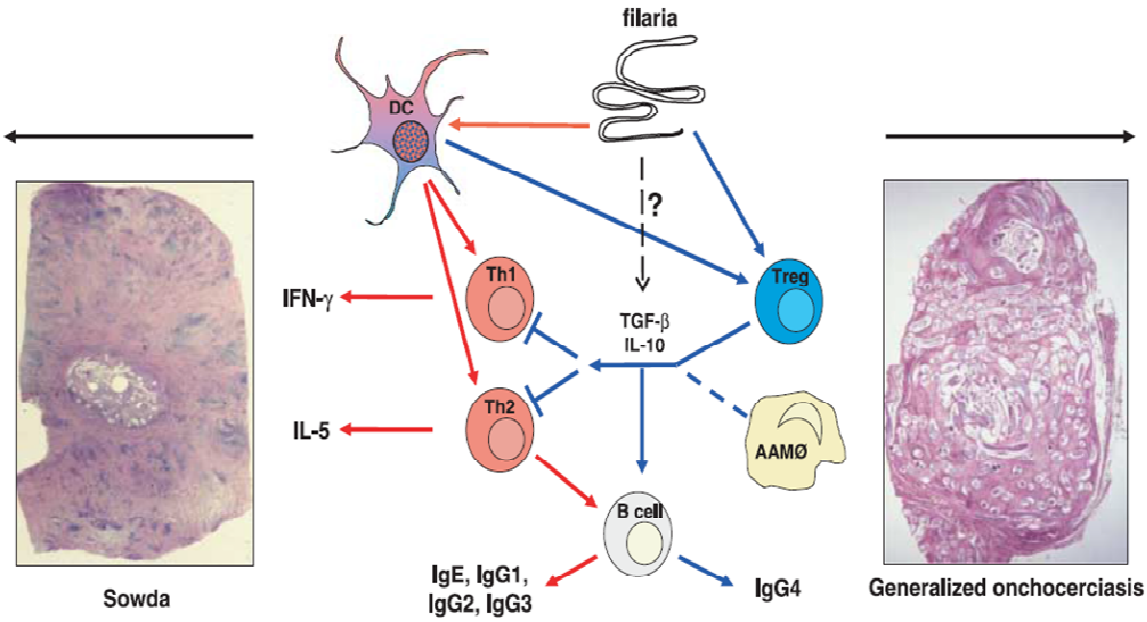

Figure 1.4: Immunity networks during onchocerciasis.

Network of interactions in the immunomodulation of filarial infection, exemplified for the polar forms of host reactivity in human onchocerciasis; generalized onchocerciasis vs. sowda (hyper-reactive onchocerciasis): Pro-inflammatory immune responses (network in red arrows) consist of Th1 and Th2 responses, with IgG 1–3 and IgE. If these reactions are not down-regulated, further effector cells such as eosinophil granulocytes, neutrophils and pro-inflammatory macrophages (not shown) are recruited and attack the adult worms and microfilariae, resulting in few adult worms in the subcutaneous onchocercomas but a high amount of inflammatory cells (sowda). In the majority of cases, down-regulatory mechanisms (network in blue arrows) comprise regulatory T cells and alternatively activated macrophages, aided by dendritic cells that are functionally impaired after encounter of filarial larvae, and potentially functionally active worm-derived TGF-beta. Production of IL-10 by these cells may not always be needed to down-regulate Th1 and Th2 effector cells, but could increase IgG4 production by B cells. These mechanisms result in generalized onchocerciasis, characterized by low pro-inflammatory reaction, allowing for the presence of high parasite loads (MF as well as many worms relative to inflammatory cells in the onchocercomas). Similar pathways exist for other filarial species [113].

1.3. Immunomodulation and immunopathology in human onchocerciasis

Immunity to onchocerciasis can be divided into three different types: (i) protective immunity without vaccination designated putative immunity, (ii) vaccine-induced immunity against L3 larvae, (iii) hyperreactive immune responses leading to microfilariae destruction and immunopathology and (iv) regulatory immune responses in patently-infected people [7]. Thus, the infection outcome ranges

across a large group of mild symptoms in people with high loads of bloodstream microfilariae (GEO) to a minority of severe pathological symptoms in HO [7, 8]. The next section will focus on the immunomodulation and immunopathology during human onchocerciasis**.**

1.4. Immunomodulation

The clinical manifestations of the disease is well illustrated in long-term exposed individuals. GEO individuals show elevated levels of immunoregulatory cytokines and cells types [117, 118]. Typical characteristics of the observed immunosuppression include antigen-specific hyporesponsiveness in T cells [119], increased IL-10 secretion which consequently increased IgG4 production in B cells [112, 120]. IgG4 is non cytolytic antibody that can compete with IgE and other IgGs for the Fc receptor binding site, therefore inhibiting degranulation of granulocytes as well as ADCC [121, 122]. The cellular basis of this immunomodulation observed in filariasis included elevated IL-10-producing $CD25^+$ Tregs, $CD4^+$ T cells that expressed high levels of glucocorticoid-induced TNF receptor family-related protein (GITR) and cytotoxic T lymphocyte antigen 4 (CTLA-4) [123, 124], alternatively activated macrophages (AAM) with anti-inflammatory properties [79]. This immunoregulation induced by *O. volvulus* compromises effector immunity but also protects the host from damaging immunopathology reactions [8].

1.5. Immunopathology

The pathological reaction and tissue damage observed in filarial infections is thought to be linked to the immune responses against the parasite [4, 23]. In lymphatic filariasis for example, Th1 and Th17 responses were observed to be associated with the lymphedema [8, 116]. In schistosomiasis, Th1 and Th2

responses were associated with hepatic granuloma formation [125, 126] while higher frequencies of Th17 cells were observed in *S. haematobium*-infected children [115]. In human onchocerciasis, patients with pronounced Th2 responses have reduced microfilaria load [7]. However, enhanced production of Th2-related cytokines and immunoglobulins such as IgE was observed in hyperreactive onchocerciasis and sowda [127]. The implication of Th17 responses in the pathological outcomes of human onchocerciasis remains to be elucidated.

1.6. Aim of the study

The pathology and immunity in populations exposed to *Onchocerca volvulus* infection is very broad. At one extreme there are individuals heavily infected with no or little pathology associated with regulatory responses and at the other extreme are hyperractive amicrofilaraemic individuals with severe pathology associated with higher Th2 responses. Th17 cells have been described as a distinct subpopulation of helper T cells with potent inflammatory properties and are characterized by their production of interleukin 17 (IL-17A and IL-17F). While the role of Th1, Th2, Treg, B cells and other innate cells has been well characterized in onchocerciasis patients, it is still unclear whether Th17 cells are involved in this pathology, therefore the expression patterns of Th1, Th2, Treg, Th17, B cells, $CD8^+$ T cells, monocytes and NK cells in peripheral blood mononuclear cells (PBMCs) from endemics normal, generalized onchocerciasis and hyperreactive onchocerciasis was examined by FACS analysis. Moreover, filarial-specific cytokine responses in an *in vitro* cell culture system after stimulation with either OvAg or αCD3/αCD28 were investigated. The cytokines and antibodies from supernatants were analyzed by ELISA and FlowCytomix multiplex system. To confirm our data at the molecular level, gene expression of GEO and HO was

done using a PCR array. In order to investigate the modulation of Th17 response in GEO individuals, an *in vitro* system was setup by inducing Th17 cells polarization either in the presence or absence of OvAg. To further assess the part played by innate responses in the differential expression of Th17 responses, PBMCs from the study groups were activated with TLRs and NLRs ligands.

2. Materials and Methods

2.1. Materials

2.1.1. Patients and *Onchocerca volvulus* infection free volunteers

In 2011, blood samples were collected from *O. volvulus*-infected individuals and included both generalized onchocerciasis and hyperresponsive onchocerciasis forms. Samples were obtained from individuals living in an *Onchocerca volvulus* endemic region of Ghana (Dunkwa). Recruited individuals included adult men and women aged 18-55 who were recruited as part of the study entitled "Enhanced Protective Immunity Against Filariasis (EPIAF)", (http://www.filaria.eu/projects/projects/epiaf.html). Ethical clearance was given by the Committee on Human Research Publication and Ethics at the University of Science and Technology in Kumasi, Ghana and the ethics committee at the University Hospital of Bonn, Germany. For comparison, samples were collected from 16 *O. volvulus* infection free volunteers from the same area and are thus referred to here as endemic normals. Written informed consent was obtained from all individuals.

2.1.2. Healthy donors

To investigate the mechanisms underlining Th17 cell differentiation and its modulation by adult *Onchocerca volvulus* worm, blood samples were collected from healthy European donors and were kindly provided by the Institute for Experimental Haematology and Transfusion Medicine, University Hospital of Bonn, Germany. Ethical approval was obtained from the University Hospital of Bonn ethics committee.

2.1.3. Plastic and glassware

Plastic and Glassware used in this study were obtained from either Eppendorf AG (Hamburg, Germany), Greiner (Frickenhausen, Germany) or Nunc (Roskilde, Denmark).

2.1.4. Antibodies, microbeads and recombinant human proteins

For *in vitro* studies, cytokines were obtained as purified recombinant human (rh) preparations: rhIL-1β and rhIL-6 were obtained from Immuno Tools (Friesoythe, Germany), rhIL-4, rhIL-10 and rhIL-4 from eBioscience (San Diego CA, USA). Human T-cell activator, anti-human CD3 and anti-human CD28 monoclonal antibody microbeads (αCD3/αCD28) were obtained from Invitrogen (Carlsbad, USA). All conjugated antibodies used for flow cytometry (FACS): anti-human CD4-APC, CD3-PerCP-Cy5.5, CD16-FITC, CD56-PE, CD45RO-FITC, CD45RA-PerCP-Cy5.5, CD8-APC, CD19-APC, CD27-FITC, CD25-PECy7, CD14-FITC, Foxp3-FITC, T-bet-PE, GATA3-PE, RORC2-PE, IFN-γ-FITC, IL-4-FITC, IL-10-PE and IL-17-FITC were obtained from eBioscience.

2.1.5. *Onchocerca volvulus* adult worm antigen extracts (OvAg)

A soluble antigen extract of *Onchocerca volvulus* worms was prepared as previously described [51]. Briefly, worms were isolated from nodules from infected people and frozen in liquid nitrogen. Frozen adult male and female worms were thawed and washed in petri dishes using 1xPBS (PAA, Pasching, Austria). Worms were then placed in a glass mortar (VWR, Langenfeld, Germany) and re-suspended in 4ml of RPMI 1640 medium (PAA) without supplements and crushed until the solution was homogenous. The extract was then centrifuged for 10 min at 300xg (4°C). The supernatant was then carefully transferred into a 50ml falcon tube. To determine the protein concentration, a Bradford Assay (see section

2.2.10.2) was performed (Advanced Protein Assay™, Cytoscelecton, Denver, USA) and the extract was aliquoted and stored at -80°C until used. All steps were performed on ice under sterile conditions. Titration of the extract was done to determine the optimal concentration for cells stimulation.

2.2. Methods

2.2.1. Parasitological assessment

Clinical diagnosis of *O. volvulus* infection was performed by examining suspected nodules and the skin. All infected individuals presented at least one nodule (onchocercomata) and skin lesions. All participants were screened for the presence of microfilariae (MF/mg skin). In short, two skin biopsies (1-3mg) from the upper part of each buttock were removed using a corneoscleral (Holth) punch (Koch, Hamburg, Germany), weighed with a Sartorius® electronic balance (Göttingen, Germany) and incubated in 100µl NaCl (0.9%) overnight at room temperature in 96 well microtiter plates. Thereafter, MF were diagnosed and counted at 63-fold magnification using an optical microscope (Leica Microsystems GmbH, Wetzlar, Germany) [43, 44]. MF load was calculated per mg of skin. Blood smears from all patients were screened for malaria infections using standard Giemsa staining protocols. Co-infections by other intestinal helminths and/or protozoa were diagnosed using standard methods on fresh stool and urine samples collected from all participants. Helminth eggs were investigated using the Kato-Katz thick smears technique [51]. Samples of individuals free of all other helminths infections and malaria were used.

2.2.2. Isolation and freezing of peripheral PBMCs from endemic normals and *Onchocerca volvulus*-infected individuals

Venous blood was collected as previously described by *Arndts et al.* [51]. Thereafter, PBMCs were isolated using a ficoll gradient centrifugation method (LSM Lymphocyte, PAA) [128, 129]. In brief, 7ml of blood was transferred into Ficoll containing tubes (Greiner) and centrifuged for 20min at 800xg at room temperature. The resulting white cell layer was transferred into a new 50ml falcon tube and washed twice with sterile endotoxin-free PBS for 8 min at 400xg at room temperature and re-suspended in RPMI 1640 supplemented with 10% FCS (PAA) and 50µg/ml of gentamycin (PAA). PBMCs were then cryo-preserved and stored in liquid nitrogen until required [111].

2.2.3. Isolation and freezing of PBMCs from healthy European donors

PBMCs from healthy donors were separated from buffy-coats using the ficoll gradient centrifugation method described in section 2.2.2 with the following modification: 35ml of PBS diluted blood was added to a 50ml Falcon tube containing 15ml ficoll.

2.2.4. Thawing of frozen cells

Cryo-tubes containing frozen PBMCs from healthy donors were removed from liquid nitrogen and quickly thawed by rubbing the tube between the palms of both hands. Cells were then pipetted into a sterile 15ml falcon tube and washed twice with 10ml cell culture medium. Thereafter, the supernatant was discarded and the cell pellet was resuspended in medium and counted as described above. All steps were performed on ice.

2.2.5. Cell viability and counting

After isolating PBMCs, cell viability was determined using trypan blue (Sigma-Aldrich) staining approach. In short, cells were diluted 1:10 or 1:100 with 0.4% trypan blue. Thereafter, 10µl of diluted cells were counted using a cell counting haemocytometer (LO Laboroptik GmbH, Bad Homburg, Germany). Unstained cells (living cells) were counted and cell numbers were estimated per ml.

2.2.6. Magnetic cell sorting of $CD4^+$ T cells, $CD4^+CD25^+$ regulatory T cells, $CD19^+$ cells and $CD14^+$ cells

In order to investigate the pathway used by OvAg to suppress Th17 cell polarization, $CD4^+$ T cells, monocytes ($CD14^+$) and B cells were isolated using specific microbeads and magnetic cell sorting technology (MACS) (Milteny Biotec GmbH (Bergisch Gladbach, Germany). To study the plasticity of Treg to Th17 cells, regulatory T cells and B cells were also isolated using the same approach. All steps were performed according to the manufacturer's instructions. $CD4^+$ T and $CD19^+$ B cells were separated by negative selection and $CD14^+$ monocytes by positive selection. $CD4^+CD25^+CD127^{dim}$ regulatory T cells were isolated in a two-step procedure using the $CD4^+CD25^+CD127^{dim}$ Reg. T cell isolation kit II. In short, the non-$CD4^+$ and $CD127^{hi}$ cell fractions were first depleted followed by the positive selection of $CD4^+CD25^+CD127^{dim}$ regulatory T cells. The purity of the isolated cells was checked by flow cytometry using the BD FACS Diva™ (BD Biosciences, Heidelberg, Germany) as described in the following section.

2.2.7. Cell characterization by flow cytometry

To address immune cell profiles in PBMCs from Ov-infected individuals, stimulated cells and to determine the purity of isolated cell types, a Fluorescence Activated Cell analysis (FACS) was performed. Flow cytometry is a laser based technology

to measure the phenotypic characteristics of cells. It allows a multi-parametric analysis of the physical and/or chemical characteristics of single cells by passing an optical and/or electronic detection apparatus. In brief, flow cytometry includes three main subsystems, the fluidic, the optic and the electronic systems. Cells of interest were solved in a fluid solution to bring the cell in the right spot for the interrogation. The optic system provides an excitation source and therefore generates and collects light signals. These optical signals were converted to proportional digital signals in the electronic subsystem.

2.2.7.1. Characterization of surface markers

All reagents were obtained from eBioscience (see section 2.1.4.). Staining was done as previously described [130]. To determine the frequency of different lymphocyte populations in *Onchocerca volvulus*-infected individuals and endemic controls, 1×10^5cells/100 µl staining buffer were incubated in FACS buffer for 30 min at 4°C with either 1) a cocktail containing anti-human CD3-PerCP-Cy5.5 (clone: OKT3), CD16-FITC (clone: CB16) and CD56-PE (clone: CMSSB) for NK and NK T cells; 2) a mixture of anti-human CD4-APC (clone: OKT4), anti-CD45RO-FITC (clone: UCHL1) and anti-CD45RA-PerCP-Cy5.5 (clone: HI100) to distinguish naïve and memory $CD4^+$ T cells.; 3) CD8-APC (clone: SK1) and CD14-FITC (clone: 61D3) for CD8 T cells and monocytes respectively or 4) CD19-APC (clone: HIB19) and CD27-FITC for memory B cells. CD14-FITC, CD19-APC, CD4-PE, Foxp3-FITC and CD25-PE-Cy7 were used to assess the purity of isolated monocytes, B cells, T cells and regulatory T cells respectively for other *in vitro* based co-culture assays.

2.2.7.2. Characterization of intracellular markers

To assess levels of transcription factors and cytokines produced by helper T cell subsets, cells were activated with a "Cells stimulation cocktail" purchased from eBioscience, which contained Phorbol 12-myristate 13-acetate (PMA), Ionomycin and protein transport inhibitor (Brefeldin A and Monensin) for 6 hours. Thereafter, surface staining was performed using anti-human CD4-APC (clone: OKT4) and cells were split into 5 groups: single staining of $CD4^+$ T cells to observe background cytokine production levels and to navigate the gating strategy; stained for Th1, Th2 and Th17 or additionally surface-stained with CD25-PECy7 (clone: BC96) to determine regulatory T cells. After a 30min incubation at 4°C, cells were washed once in FACS buffer. After fixation and permealibization in 1/4x Fix-Perm reagent (eBioscience), cells were incubated at 4°C for 30 minutes with either 1) anti-human T-bet-PE (clone: eBio4B10) and IFN-γ-FITC (clone: 4S.B3) for the Th1 panel; 2) GATA3-PE (clone: TWAJ) and IL-4-FITC (clone: B-S4) for Th2 cells; 3) RORC2-PE (clone: AFKJS-9) and IL-17A-FITC (clone: eBio64DEC17) for a Th17 phenotype and or 4) Foxp3-FITC (clone: 236A/E7) and IL-10-PE (clone: JES3-9D7) for regulatory T cells. Thereafter, cells were washed twice and re-suspended in fix-perm buffer (eBioscience). To avoid unspecific binding, Fc block (eBioscience) was used and for spectral overlap correction, fluorescence compensation was done using UltraComp ebeads (eBioscience). Data were acquired and analyzed on a FACS Canto flow cytometer (BD Biosciences). The gating strategy is depicted in Figure 2.1.

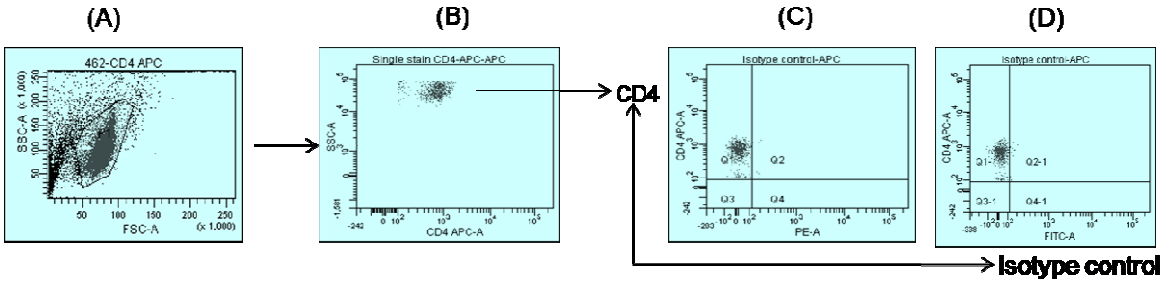

Figure 2.1: Gating strategy.

(A) lymphocyte gate and *(B)* $CD4^+$ T cell gate from total lymphocytes. (*C)* and (*D)* are the isotype controls for respectively PE and FITC staining.

2.2.8. Cytokine and immunoglobulin multiplex assays

The levels of cytokines and immunoglobulins from resulting cell culture supernatants were measured using the FlowCytomix Multiplex system following the manufactures instructions (eBioscience). For cytokines a human-specific 13-plex cytokines kit was selected which detected IL-12p70, IL-1β, IL-2, TNF-α, IFN-γ IL-6, IL-5, IL-4, IL-9, IL-10, IL-13, IL-17A and IL-22. To determine levels of immunoglobulins, a human specific 6-plex kit was chosen which detected IgG1, IgG2, IgG3, IgG4, IgA and IgM. In addition a simplex kit for IgE was also used. Measurements were determined using the BD FACS Canto^{TM} flow cytometer (BD Biosciences).

2.2.9. *In vitro* co-culture assays

2.2.9.1. Onchocerca volvulus antigen-specific responses in infected individuals

To study levels of immune responsiveness to *Onchocerca volvulus* adult worm antigen, isolated PBMCs from EN, GEO and HO individuals were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin (100µg/ml), Streptomycin (100µg/ml), gentamycin (50µg/ml) and 5ml L-glutamine (292.3µg/ml). For *in vitro* analysis, $1x10^5$ PBMCs were plated in 96 round bottomed well plates (U-shaped, Greiner Bio-one) and either left unstimulated for spontaneous background cytokine

and immunoglobulin secretions or stimulated with either OvAg (20µg/ml) or αCD3/αCD28 beads (40,000beads/well, Invitrogen). Samples were cultured in duplicate for 7 days to assess cytokine secretion and in unicate for 14 days for immunoglobulin analysis. Supernatants were pooled for cytokines analysis.

2.2.9.2. In vitro modulation of Th17 cell generation by OvAg assay

To investigate the modulation of Th17 cells development and differentiation by OvAg, isolated PBMCs from healthy donors were plated at a concentration of $2x10^5$ cells/well. These cells were then stimulated with αCD3/αCD28 (20,000 beads/well were use for this experiment) in the presence of a chemokine cocktail (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml) or both the CC and OvAg (12.5µg/ml). Cells were cultured for 7 days at 37°C under 5% CO_2.

In order to assess the soluble factors underlining the observed modulatory effect, PBMCs were further cultured and activated with αCD3/αCD28 in combination with either (i) the CC or (ii) the CC in the presence of rhIL-4, rhIL-13 and rhIL-10 or (iii) the CC and OvAg in the presence or absence of neutralizing antibodies for anti-hIL-4, anti-hIL-13 and anti-hIL-10. Cells were incubated for 7 days under the same conditions.

To investigate cellular mechanisms that are involved in the suppressive effect of OvAg, $1x10^5$ $CD4^+$ T cells were cultured alone or in co-culture with either $1x10^5$ B cells or $1x10^5$ monocytes per well. T cells were then activated with αCD3/αCD28 in the presence or absence of the CC. After 7 days of culture, supernatants were removed and the cytokine content was measured by enzyme-linked immunosorbent assay (ELISA, as described in section 2.2.10.1).

2.2.9.3. In vitro study of the shift of GEO phenotype to HO phenotype

To investigate the potential transformation of a GEO phenotype into a HO phenotype, PBMCs from EN and GEO individuals were stimulated with C12-iE-DAP (PGN-like molecule, 100ng/ml) a NOD1 ligand, ODN 2006 (TLR9 ligand, 1μM), LPS (TLR4 ligand, 10ng/ml) and Muramyl dipeptide (10μg/ml) a NOD2 ligand for 7 days. To assess the soluble factors involved in this transformation, cells from EN and GEO were left unstimulated or stimulated with αCD3/αCD28 in the presence or absence of recombinant human IL-6 and IL-1β (CC). The resulting supernatant was then analysed for the presence if IL-17A, IFN-γ., IL-4 and IL-10 by Flowcytomix multiplex assay as described in section 2.2.8.

2.2.10. Proteins and molecular biology assay

2.2.10.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokines in cell culture supernatants were quantified using ELISA. Cytokines such as IL-17A, IL-4, IL-10, IL-13 and IFN-γ were measured from the Th17 cells modulation experiments and levels of IL-6, TNF-α and IL-10 in the plasticity Treg-Th17 experiments. Briefly, after diluting the specific capture antibody (1:250) in coating buffer (eBioscience), ELISA plates were coated (50µl/well) with capture antibody and incubated overnight at 4°C. After 5 washing steps (wash buffer appendix), plates were blocked with assay diluents (eBioscience) 50µl/well, for an hour at room temperature. Thereafter, plates were washed as previously stated and standards and samples were added (50µl/well) and incubated overnight at 4°C. Secondary biotinylated antibodies (Detection Antibody) were diluted 1:250 and added to the plates for 1hr at room temperature. Following a further 5 times washing, 50 µl/well of streptavidin-peroxidase, diluted (1:250), was added to the plates and incubated in darkness at room temperature for 30 minutes. After, plates

were washed a final 7 times, 50µl/well of Tetramethybenzidine (TMB, eBioscience) substrate was added. Lastly, to stop the reaction 30µl/well of $2NH_2SO_4$ was added. Plates were measured at 450nm using a plate reader SpectraMAX 340 Pc (Molecular Devices, Ismning, Germany). Data were analyzed with SoftMax Pro 5.0 software (Molecular Devices).

2.2.10.2. BradFord protein assay

To measure the protein concentration of OvAg extract a Bradford protein assay was performed as described by the manufacturer's protocol. In brief, serial dilutions of bovine serum albumin (BSA) was performed and used as standards against the samples (OvAg). Another serial dilution of the samples was done in PBS. 300µl per well of Coomassie blue G (Cytoscelecton, Denver, USA) reagent was distributed in duplicate in an ELISA plate and 3µl of diluted samples and standard were added accordingly. The protein concentration was quantified at 595 nm using a plate reader SpectraMAX 340 Pc (Molecular Devices).

2.2.10.3. RNA Isolation, Reverse Transcription and Real Time PCR (qPCR) array

To investigate in depth the differences between GEO and HO, 84 genes were screened by PCR Array using the RT^2SYBR Green Mastermix kit (Qiagen, Hilden, Germany) and RT^2 Profiler PCR Array Human Th17 for autoimmunity and inflammation kit (Qiagen) according to the manufacturer's protocol. This array profiles genes related to: i) Surface Molecules: CD28, CD34, CD4, CD8A, ICAM1, ICOS, TLR4, ii) Chemokines: CCL1 (I-309), CCL2 (MCP-1), CCL20 (MIP-3A), CCL22 (MDC), CCL7 (MCP-3), CX3CL1, CXCL1, CXCL12 (SDF1), CXCL2, CXCL5 (ENA78/LIX), CXCL6 (GCP-2), IL8, MMP3, MMP9, iii) Cytokines: CSF2 (GM-CSF), CSF3 (GCSF), IFNγ, IL10, IL12B, IL13, IL15, IL17A, IL17C, IL17D, IL17F, IL18, IL1B, IL2, IL21, IL22, IL23A, IL25 (IL17E), IL27, IL3, IL4, IL5, IL6, IL9,

TGFB1, TNF, iv) Cytokine Receptors: CCR2, CCR4, CCR6, IL1R1, IL12RB1, IL12RB2, IL17RA, IL17RB, IL17RC, IL17RE, IL23R, IL6R, IL7R and v) Signaling Pathway Molecules and Transcriptional Factors: CD40LG (TNFSF5), CEBPB, CLEC7A (Dectin-1), FOXP3, GATA3, IRF4, ISG20, JAK1, JAK2, NFATC2, NFKB1, RORA, RORC, RUNX1 (AML1), S1PR1, SOCS1, SOCS3, STAT3, STAT4, STAT5A, STAT6, SYK, TBX21, TRAF6. In brief, PBMCs from GEO, EN and HO individuals were stimulated with αCD3/αCD28 (Ratio beads-to-cell 1:10) for 3 hours at 37°C. Thereafter RNA was extracted using Trizol (Invitrogen). DNA was digested using the DNA-free kit (Invitrogen) and the concentration and the purity of RNA was determined using the NanoDrop 1000 (Peqlab, Erlangen, Germany). Extracted RNA was reverse transcribed using the Qiagen Master mix (Qiagen,) by adding 0.5µg of RNA and incubating in the Primus Thermocycler (MWG-Biotech, Ebersberg, Germany). Amplification was performed on the RotorGene 6000 (Corbett Research, Sydney, Australia). Data were analyzed by RT^2 profiler PCR Array data analysis 3.5 software (Qiagen).

2.2.11. Statistical analysis

Statistical analyses were performed using the software PRISM 5.02 (GraphPad Software, Inc., La Jolla, USA). Depending on the parameter distribution of the data sets, statistical differences were observed using either an ANOVA or Kruskal-Wallis-test and, if significant, followed by Student's t test or Mann-Whitney–U test for a further comparison of two groups. P-values of 0.05 or less were considered significant * p<0, 05, ** p<0, 01 and *** p<0, 001.

3. Results

3.1. Population characteristics

Onchocerca volvulus-infected individuals within this study cohort were diagnosed as described in section 2.2.2. Individuals were then further characterized as having either generalized onchocerciasis (GEO, n=28) or hyperreactive onchocerciasis (HO, n=6). For comparison, infection-free volunteers, residing in the same area were recruited as well (EN, n=28). Table 1 presents the characteristics of the study population. The mean age was similar in each study group (38.96 vs. 38.66 in GEO and HO respectively). The number of male and female participants in the HO group was equal and in the GEO and EN groups they were comparable. Microfilariae load, the number of nodules and sites of infection were only described for the GEO group. HO individuals presented neither microfilariae nor nodules. HO individuals presented either depigmentation/leopard skin condition (n=3); acute papular onchodermatitis (n=1) or sowda (n=2). The immunological determinant which drives the differences between GEO and HO needs further investigations. Therefore, in the following section, immune cell profiles in GEO and HO versus EN was characterized.

Table 1: Characteristics of the study population

	GEO (n=28)	HO (n=6)	Endemic normals (n=28)
Age (mean/range)	38.96 (19-55)	38.66 (24-55)	38.26 (18-55)
Gender (F/M)	11/17	3/3	15/13
Mf load mg/skin	8.99 (0.1-86.10)	0	-
Number of nodules	1.97(1-9)	0	
Number of sites	1.36(1-6)	0	
Dermatitis	-	Yes	
Co-infections	0	0	0

Age: mean (range); Gender: Number of female/male participants; Mf load mg/skin count: mean (range); Number of nodules: mean (range); Number of sites: mean (range), Co-infection: Number of co-infected. GEO: Generalized onchocerciasis; HO: Hyperreactive onchocerciasis.

3.2. Differing immune cell profiling in *Onchocerca volvulus*-infected individuals

3.2.1. Higher frequencies of monocytes and memory helper T cells

To distinguish whether differences in immune cell profiles occur in individuals presenting the polar forms of onchocerciasis, the frequency of NK cells, NK T cells, monocytes, B cells, T helper cells and $CD8^+$ T cells from PBMCs were analyzed. With regards to the latter population, individuals with GEO had significantly less $CD8^+$ T cells when compared to EN (Figure 3.1A). NK and NKT cell populations were determined using combinations of anti-CD3, anti-CD16 and anti-CD56 (Figure 3.1B-E).

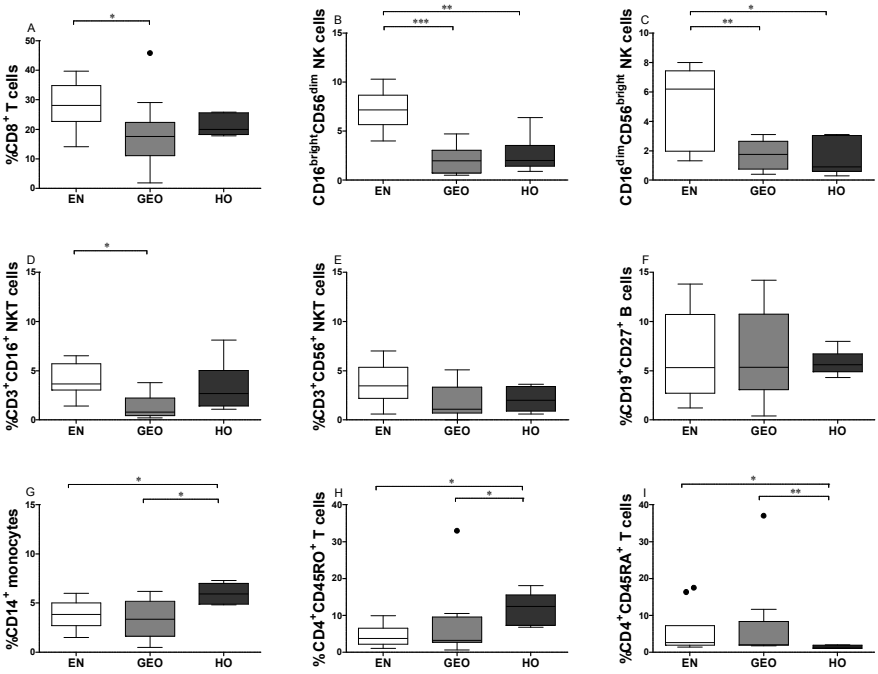

Figure 3.1: Evidence of reduced frequencies of monocytes and memory helper T cells in individuals with GEO onchocerciasis.

Isolated PBMCs from EN (n=16) and individuals presenting either generalized (GEO, n=16) or hyperreactive onchocerciasis (HO, n=6) were stained with a combination of antibodies to determine the frequencies of $CD8^+$ T cells (A); NK cells [$CD3^-CD16^{bright}CD56^{dim}$ (B) and $CD3^-CD16^{dim}CD56^{bright}$ (C)]; NKT cells [$CD3^+CD16^+$ (D) and $CD3^+CD56^+$ (E)]; $CD19^+CD27^+$ memory B cells (F); $CD14^+$ monocytes (G); memory $CD4^+$ T cells (H) and naive $CD4^+$ T cells (I). Graphs show box whiskers tukey with outliers from EN n=10, GEO n=10 and HO n=6. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001).

When compared to levels in EN, both $CD16^{bright}CD56^{dim}$ and $CD16^{dim}CD56^{bright}$ NK populations were significantly lower in GEO and HO individuals but no differences could be observed between GEO and HO groups (Figure 3.1B and C respectively). $CD3^+CD16^+$ but not $CD3^+CD56^+$ NKT cells were also lower in GEO but not HO individuals (Figure 3.1D and E). Whereas no differences could be observed in the frequency of memory B cells (Figure 3.1F), significantly elevated numbers of monocytes ($CD14^+$ cells) were identified in HO individuals (Figure 3.1G). This phenotype was also observed upon staining for memory $CD4^+CD45RO^+$ T cell populations (Figure 3.1H) and interestingly, levels of naive $CD4^+CD45RA^+$T cells in this group were significantly lower than in individuals with

GEO or EN (Figure 3.1I). This suggests an active immune response associated with helper T cell activity in HO individuals.

3.2.2. Hyperreactive onchocerciasis is associated with elevated $CD4^+$IL-17^+A and $CD4^+$IL-4^+-producing T cells

The increased monocyte frequencies in HO individuals in the previous section could be explained by an increased antigen presentation requirement. This could have led to the elevated memory helper T cells seen in this group (Figure 3.1H). $CD4^+$ T cells upon activation by APC can differentiate into effector Th1, Th2 and Th17 cells and iTreg [103, 104]. Therefore, we next determined the frequency of $CD4^+$ T cells producing IFN-γ, IL-4, IL-17A and IL-10 through intracellular staining after activating PBMCs with PMA, Ionomycin, Brefeldin A and Monensin (as described in section 2.2.6.2). As shown in Figure 3.2A, the frequency of $CD4^+$IFN-γ^+ T cells was significantly higher in EN than in either *O. volvulus*-infected group. Moreover, $CD4^+$ T cells from GEO individuals produced significantly higher amounts of IFN-γ than cells from HO individuals (Figure 3.2A). In contrast, individuals with GEO or HO displayed significantly elevated frequencies of IL-4-secreting $CD4^+$ T cells when compared to EN groups (Figure 3.2B). Interestingly, cells from HO individuals produced more IL-4 than cells from GEO persons (Figure 3.2B), a phenotype further reflected by $CD4^+$ T cells secreting IL-17A (Figure 3.2C). Whereas IL-17A producing $CD4^+$ T cells appeared to be a unique characteristic in individuals presenting the hyperreactive form of the disease, those with GEO had a dominant IL-10 producing phenotype (Figure 3.2D). Figure 3.3 shows representative flow cytometry data depicting the expression in percentages of Th2 and Th17 cytokine release in EN, GEO and HO individuals.

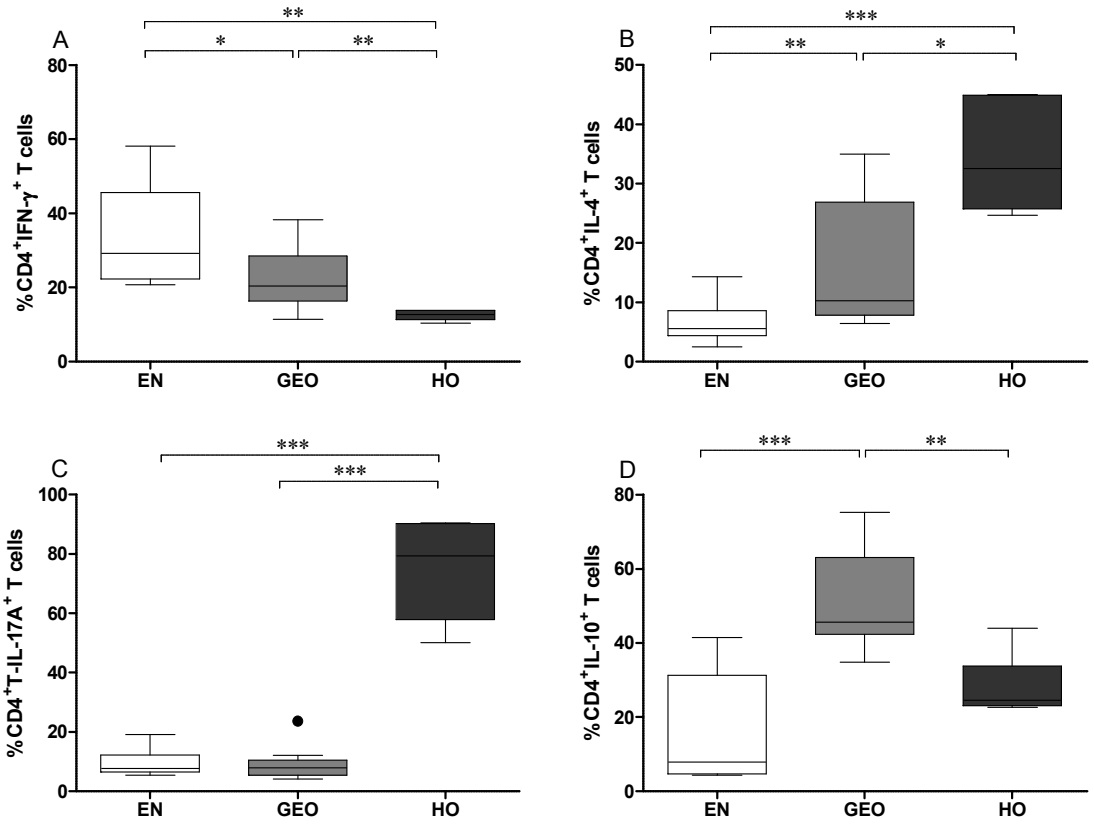

Figure 3.2: Hyperreactive individuals exhibit a dominant IL-4 and IL-17 phenotype.

Isolated PBMCs from EN (n=10) and *O. volvulus*-infected individuals presenting either GEO (n= 10) or HO (n=6) were activated with a stimulation cocktail (PMA, Ionomycin, Brefeldin A and Monensin) for 6 hours. Thereafter, cells were harvested and stained with an anti-CD4 antibody and after fixation and permeabilization with anti-human antibodies specific for IFN-γ (A), IL-4 (B), IL-17A (C) and IL-10 (D). Intracellular cytokine expression was determined on the $CD4^+$ T cell population by flow cytometry. Graphs show percentages as box whiskers with outliers. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001).

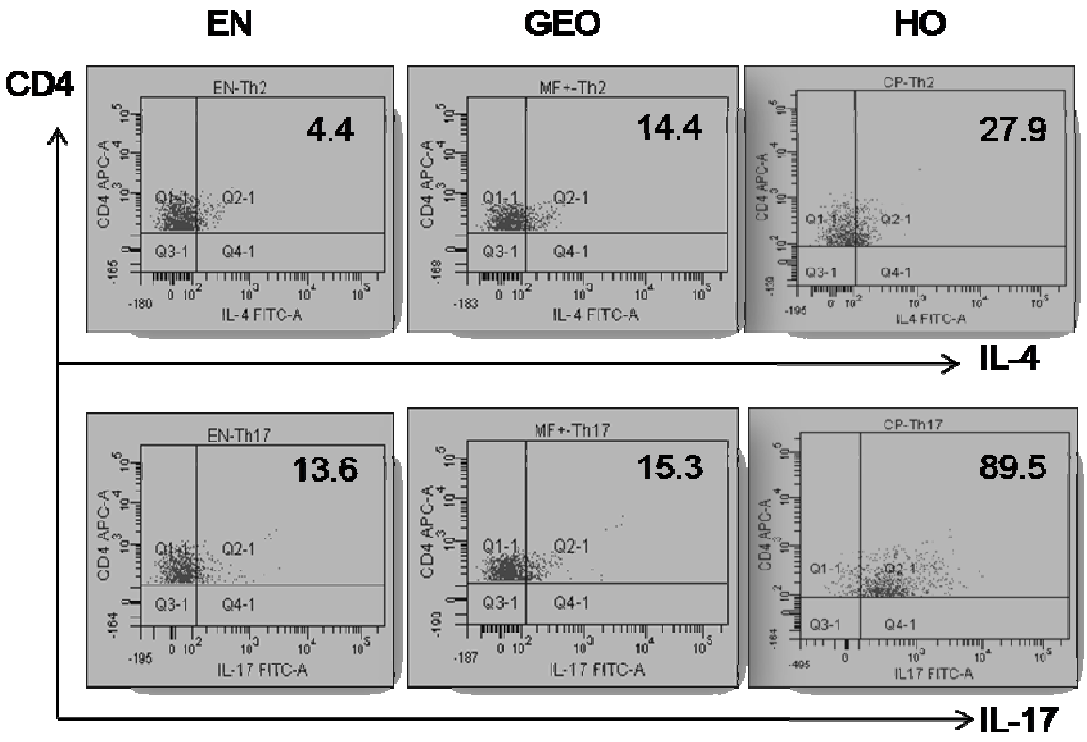

Figure 3.3: Expression profiles in percentage of different Th2 and Th17 cell cytokine in PBMCs from EN, GEO and HO individuals.

PBMCs from EN (n=10) and *O. volvulus*-infected individuals presenting either GEO (n= 10) or HO (n=6) were activated with a stimulation cocktail (PMA, Ionomycin, Brefeldin A and Monensin) for 6 hours. Thereafter, cells were harvested and stained with an anti-CD4 APC antibody and either IL-4 or IL-17A conjugated antibodies. Each dot blot represents the cytokine expression in a representative individual from the EN (left panel), the GEO (middle) and the HO (right) groups. Flow cytometry was performed using a BD FACS Canto I and analyzed using FACSDiva 5.2 software.

3.2.3. Higher expression of $CD4^+GATA3^+$ and $CD4^+RORC2^+$ T cells in hyperreactive individuals

In the above section the cytokine expression profiles in the HO group showed dominant Th2 and Th17 responses. To compare the frequencies of $CD4^+$ T cells with related transcription factors and correlate them with the cytokine profiles, the expression of T-bet, GATA3, RORC2 and Foxp3 was also assessed by flow cytometry. In association with their elevated amounts of IFN-γ-producing $CD4^+$ T cells (Figure 3.2A), EN had significantly higher numbers of $CD4^+$T-bet^+ T cells when compared to hyperreactive onchocerciasis individuals (Figure 3.4A).

Strikingly, the Treg associated transcription factor Foxp3 was prominently expressed by $CD4^+$ T cells from hyperreactive individuals, even when compared to individuals with GEO (Figure 3.4B).

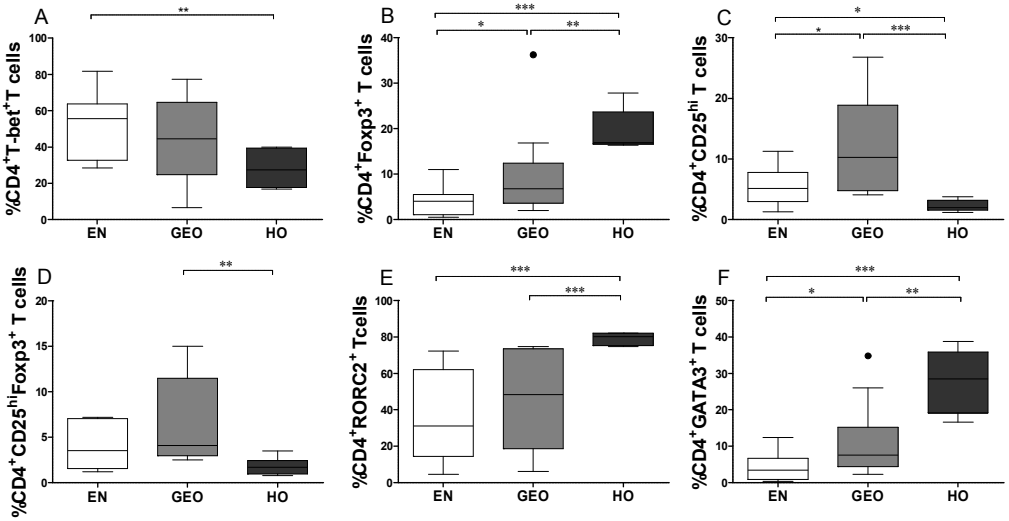

Figure 3.4: Hyperreactive onchocerciasis individuals present higher frequencies of $CD4^+Foxp3^+$ but not $CD4^+CD25^{hi}Foxp3^+$ T cells.

PBMCs from EN and *O. volvulus*-infected GEO or HO individuals were activated with a cell stimulation cocktail (PMA, Ionomycin, Brefeldin A and Monensin) for 6 hours. Thereafter, cells were harvested and stained for CD4 and the transcription factors, T-bet (A), Foxp3 (B,D), RORC2(E) and GATA3(F). PBMCs fractions were then further stained with CD25 to assess the numbers of $CD4^+CD25^{hi}$ (C) and $CD4^+CD25^{hi}Foxp3^+$ (D) T cells in each individual (EN n=16, GEO n= 16, HO n= 6). Cell population frequencies were determined via flow cytometry. Graphs show percentages as box whiskers with outliers. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001).

To elucidate on this unexpected expression of Foxp3 in HO individuals, we expanded our profile panel to include $CD4^+CD25^{hi}Foxp3^+$ T cells using the above depicted gating strategies (Figure 2.1 and Figure 3.3). Interestingly, the inclusion of $CD25^{hi}$ cells dramatically changed the profile of $CD4^+$ T cells from HO individuals since both $CD4^+CD25^{hi}$ (Figure 3.4C) and $CD4^+CD25^{hi}Foxp3^+$ (Figure 3.4D) T cell subsets were higher in GEO individuals. More interestingly, the expression of RORC2 (Figure 3.4E) and GATA3 (Figure 3.4F) correlated with IL-17A and IL-4 profiles and were higher in HO individuals compared to EN and GEO

groups. Moreover, expression levels of $CD4^+GATA3^+$ T cells were increased in GEO compared to EN participants (Figure 3.4F). These data indicate a correlation between T cell transcription factors and cytokine induction in onchocerciasis patients and confirm that Th2 and Th17 are characteristic of hyperreactive onchocerciasis.

3.2.4. Increased ratios of Th17/Treg in hyperreactive onchocerciasis individuals

The combination of Th2 and Th17 responses characterized individuals with hyperreactive onchocerciasis. In order to evaluate the dominant T helper response, ratios amongst the different T helper cytokines and transcription factors were assessed. These data revealed that there were significant increases in the ratio of $CD4^+IL\text{-}4^+$ vs $CD4^+IFN\text{-}\gamma^+$ in HO compared to EN and GEO (Figure 3.5A). Moreover, this ratio was increased in GEO compared to EN (Figure 3.5A) confirming the prominent Th1 response in EN.

Additionally, the ratio in $CD4^+IL\text{-}17A^+$ vs $CD4^+IFN\text{-}\gamma^+$ (Figure 3.5B) was increased in HO compared to EN and GEO. More interestingly, the ratios $CD4^+CD25^{hi}Foxp3^+$ vs $CD4^+GATA3^+$ (Figure 3.5C), $CD4^+RORC2^+$ vs $CD4^+CD25^{hi}Foxp3^+$ (Figure 3.5D) and $CD4^+IL\text{-}17A^+$ vs $CD4^+IL\text{-}10^+$ (Figure 3.5E) were increased in HO compared to EN and GEO individuals; confirming the dominant Th2 and Th17 responses in HO. The ratios of $CD4^+IL\text{-}10^+$ vs $CD4^+IFN\text{-}\gamma^+$ (Figure 3.5F) and $CD4^+IL\text{-}10^+$ vs $CD4^+IL\text{-}4^+$ (Figure 3.5G) were increased in GEO compared to the other groups confirming the dominant regulatory response in GEO individuals. Moreover the ratio $CD4^+RORC2^+$ vs $CD4^+T\ bet^+$ was higher in HO compared to either EN or GEO(Figure3.5H). These results suggest that the balance of Th17/Treg determine the pathological outcomes in hyperreactive onchocerciasis.

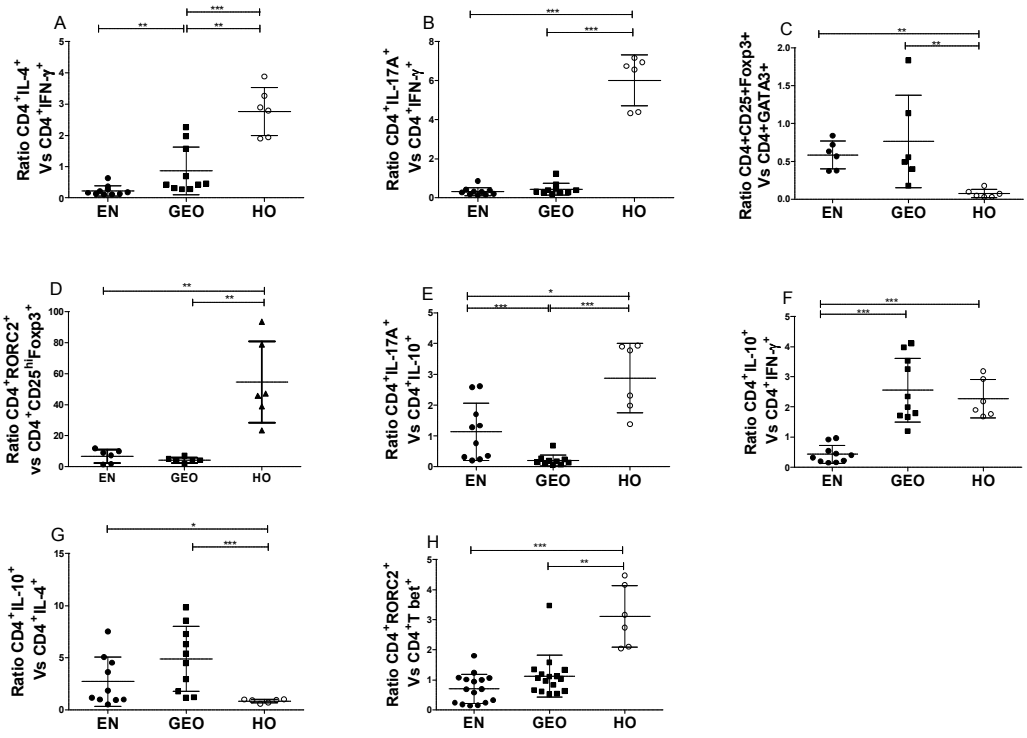

Figure 3.5: Elevated ratios of $CD4^+IL\text{-}17A^+$ vs $CD4^+IL\text{-}10^+$ in HO individuals.

Following the assessment of cytokine levels in $CD4^+$ T cells in all three study groups via flow cytometry ratios amongst the different profiles were determined. (A) $CD4^+IL\text{-}4^+$ vs CD4+IFN-γ^+, (B) $CD4^+IL\text{-}17A^+$ vs $CD4^+IFN\text{-}\gamma^+$, (C) $CD4^+RORC2^+$ vs $CD4^+GATA3^+$, (D) $CD4^+RORC2^+$ vs $CD4^+CD25^{hi}Foxp3^+$, (E) $CD4^+IL\text{-}17A^+$ vs $CD4^+IL\text{-}10^+$, (F) $CD4^+IL10^+$ vs $CD4^+IFN\text{-}\gamma^+$, (G) $CD4^+IL\text{-}10^+$ vs $CD4^+IL\text{-}4^+$ and (H) $CD4^+RORC2^+CD4^+T\ bet^+$. Each dot represents the ratio ±SD of these combinations. Data of each ratio constellation were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p<0.05, **p<0.01 and ***p<0.001.

3.2.5. Up-regulation of markers for Th2 and Th17 gene expression in hyperreactive onchocerciasis

To further investigate the association of hyperresponsiveness and elevated IL-17A- and IL-4-secreting $CD4^+$ T cells, RT-qPCR was performed using RT^2 Profiler PCR Array Human Th17 kit . This kit profiles the expression of 84 genes related to the Th17 Regulatory Network such us i) Surface Molecules: CD28, CD34, CD4, CD8A, ICAM1, ICOS, TLR4, ii) Chemokines: CCL1 (I-309), CCL2 (MCP-1), CCL20 (MIP-3A), CCL22 (MDC), CCL7 (MCP-3), CX3CL1, CXCL1, CXCL12 (SDF1), CXCL2, CXCL5 (ENA78/LIX), CXCL6 (GCP-2), IL8, MMP3, MMP9, iii)

Cytokines: CSF2 (GM-CSF), CSF3 (GCSF), IFNγ, IL10, IL12B, IL13, IL15, IL17A, IL17C, IL17D, IL17F, IL18, IL1B, IL2, IL21, IL22, IL23A, IL25 (IL17E), IL27, IL3, IL4, IL5, IL6, IL9, TGFB1, TNF, iv) Cytokine Receptors: CCR2, CCR4, CCR6, IL1R1, IL12RB1, IL12RB2, IL17RA, IL17RB, IL17RC, IL17RE, IL23R, IL6R, IL7R and v) Signaling Pathway Molecules and Transcriptional Factors: CD40LG (TNFSF5), CEBPB, CLEC7A (Dectin-1), FOXP3, GATA3, IRF4, ISG20, JAK1, JAK2, NFATC2, NFKB1, RORA, RORC, RUNX1 (AML1), S1PR1, SOCS1, SOCS3, STAT3, STAT4, STAT5A, STAT6, SYK, TBX21, TRAF6.

As described in section 2.2.10.3. RNA was isolated from PBMCs of HO (n=2) and GEO (n=2) individuals. Extracted RNA was reverse transcribed using Qiagen Master mix and incubated in the Primus Thermocycler. Amplification was performed on the RotorGene 6000. Data were analyzed using Qiagen free PCR Array Data Analysis Software. A total of 46 genes were over-expressed, 18 genes were down-regulated and 20 genes were unchanged (Figure 3.6A). In correlation with the higher amounts of Th17 cells, IL-17 gene family members such as *IL17A*, *IL17C*, *IL17D* and *IL17F* were all up-regulated in cells from HO individuals (Figure 3.6C). Furthermore, this trend was observed with Th17-related transcription factor *RORC* and signalling pathway molecule *STAT3* genes (Figure 3.6C). The genes of cytokines known to be required for the induction of Th17 cells such as IL-6, IL-1β, TGF-β1, IL-21 and IL-23A [131, 132] were also highly up-regulated in HO persons (Figure 3.6D). In addition the *IL22* gene, another Th17 related cytokine was up-regulated in hyperreactive individuals (Figure 3.6D). Correlating with the unexpected observation that $CD4^{+}Foxp^{+}$ cells were higher in HO compared to GEO (Figure 3.2), Foxp3 gene expression followed the same trend (Figure 3.6A. In contrast, suppressor of cytokine signaling protein 3 gene (SOCS3) was

downregulated in HO (Figure 3.6A. With regards to Th2-related genes, here the *IL13* gene presented the strongest fold increase and correlated to the elevated gene expression of *GATA3* and *STAT6* (Figure 3.6E). More interestingly, in correlation with elevated Treg responses observed in GEO compared to HO, Treg-related genes CCL1 and IL-7R were down-regulated in HO compared to GEO (Figure 3.6A. Cytokines profiling in Figure 3.2 showed elevated $CD4^{+}IFN\text{-}\gamma^{+}$ cells in GEO compared to HO. In concordance with this data, Th1-related genes such as IL-12B and IL-12RB genes were also up-regulated in GEO compared to HO individuals (Figure 3.6A). Overall, these findings confirm and correlate with the T helper cell profiles observed in section 3.2.2.

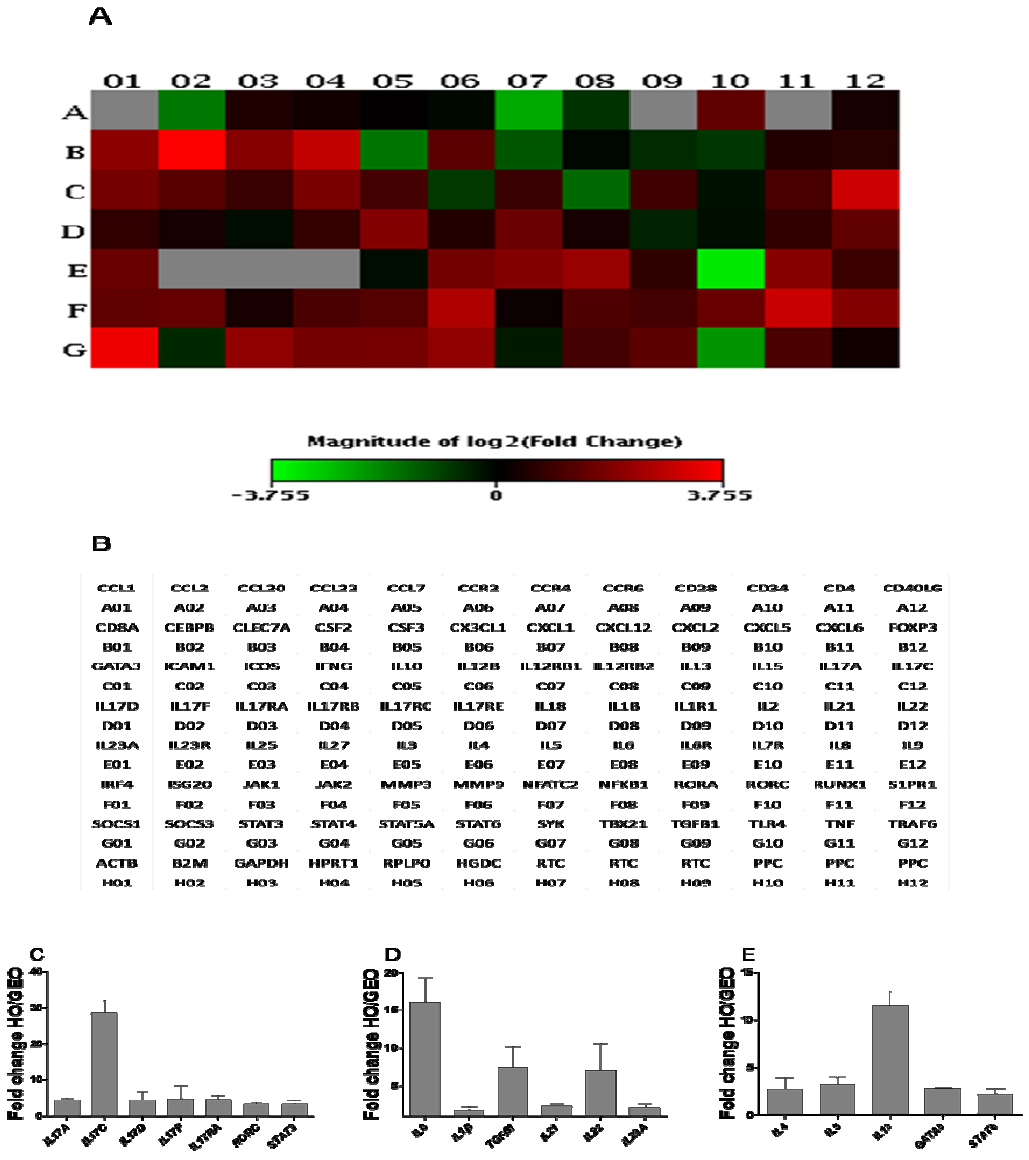

Figure 3.6: Up-regulation of Th2 and Th17 gene expression in HO individuals.

PBMCs were stimulated with αCD3/αCD28 (ratio beads-to-cell 1:10) for 3h, RNA was extracted and transcribed into cDNA. Thereafter, PCR Arrays were performed using the RT^2 Profiler PCR Array Human Th17 kit. Data were analyzed using Qiagen free PCR Array Data Analysis Software. (A), A heat map provides a graphical representation of fold regulation expression data between the two groups (GEO Vs HO). (B), Corresponding gene table. (C-E), Bars show fold change of 1.5 ± SEM increase in the indicated genes between four age-matched males presenting either GEO or HO forms of infection.

3.3. *Onchocerca volvulus* antigen-specific responses in infected individuals

Peripheral mononuclear cell profiling in section (3.2) using FACS analysis and RT-qPCR has shown that hyperreactive onchocerciasis individuals exhibited higher levels of Th2 and Th17 responses, whereas EN and GEO depicted increased levels of Th1 and Treg responses respectively. Both techniques FACS and qPCR could not tell if these different responses were filarial-specific or not. Therefore, in this section it was further investigated using *in vitro* cell cultures the specificity of these responses.

3.3.1. Elevated OvAg-specific Th1 and Th2 responses in endemic normals and hyperreactive onchocerciasis individuals respectively

In the previous sections (3.2.2 and 3.2.3), elevated Th1 and Th2 profiles were revealed in HO individuals using flow cytometry. Since helminth infections have been shown to induce Th and Th2 type response in putative immunity and infected individuals respectively, in this section, it was assessed whether these responses were also reflected in cell cultures following activation with an antigen preparation derived from *Onchocerca volvulus* adult worms (OvAg). Interestingly, when compared to either EN or GEO groups, OvAg-stimulated (20µg/ml) PBMCs from HO individuals made significantly higher amounts of IL-5 (Figure 3.7A). Upon TCR activation, no difference was seen between the groups but increased induction of this cytokine was observed in EN, GEO and HO compared to their control cultures (Figure 3.7B). Another Th2 cytokines, IL-13, produced a similar pattern to IL-5 following OvAg stimulation (Figure 3.7C). Interestingly, not only the activation with αCD3/αCD28 reflected increased levels of IL-13 in each group compared to its control but also this was statistically significant in HO compared to EN and GEO. (Figure 3.7D). This induction was similar to those observed in IFN-γ (Th1 cytokine)

levels after TCR activation (Figure 3.7E). Base line secretion of IFN-γ was higher in EN compared to HO (Figure 3.7F). Regarding IL-10 secretion, no differences were seen upon OvAg stimulation (Figure 3.7G) but a higher induction was observed in both GEO and HO compared to their controls after TCR activation (Figure 3.7H).

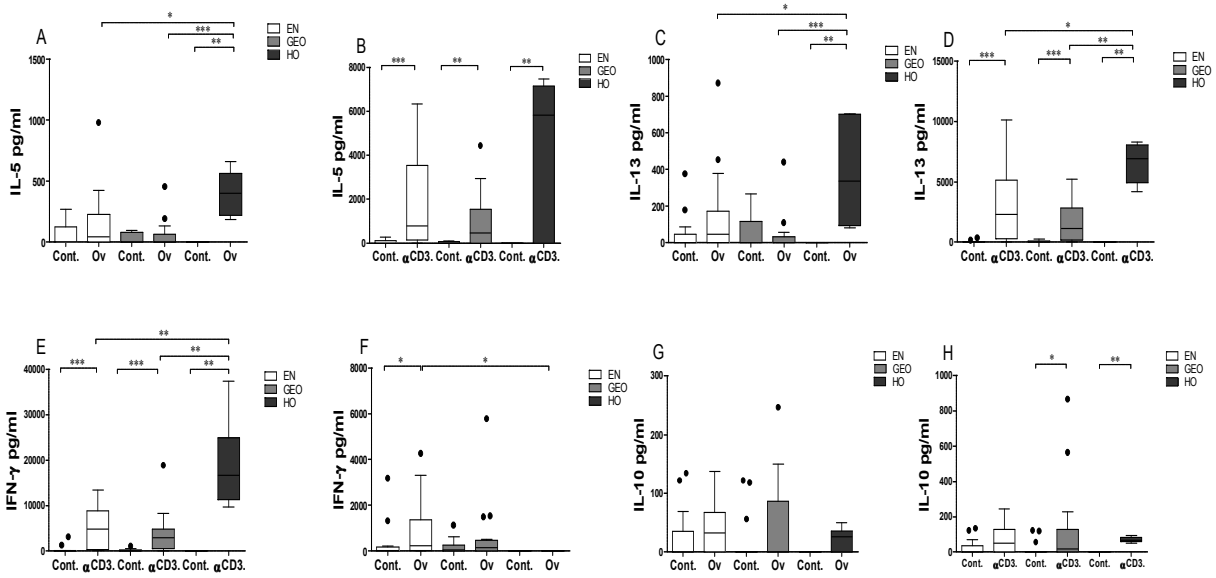

Figure 3.7: Hyperreactive individuals present stronger filarial-specific Th2 responses *in vitro*.

Isolated PBMCs (1x10^5/well) from EN (n=16) and *O. volvulus*-infected GEO (n= 16) or HO (n=6) individuals were left either unstimulated or activated with an *O. volvulus* antigen extract (OvAg, 20µg/ml) and αCD3/αCD28 (αCD3, 40,000beads/well) for 7 days. Thereafter, levels of IL-5 (A, B), IL-13 (C, D), and IFN-γ (E, F) and IL-10 (G, H) were measured in the culture supernatants using a FlowCytomix™ Multiplex kit via flow cytometry. Graphs show data as box whiskers with outliers. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001).

3.3.2. Increased Th17-related cytokines after OvAg stimulation

Th17 cell markers and their related cytokines were observed to be associated with HO using FACS and qPCR array (section 3.2.4, 3.2.5 and 3.2.6). As mentioned above, the induction of Th17 cells has been shown to require IL-6, IL-1β, TGF-β

and IL-23 [133], therefore the levels of Th17-related cytokines following activation with either OvAg or αCD3/αCD28 were also measured. Interestingly, basal levels of IL-17A production from cell cultures of HO individuals were equal to those stimulated with OvAg and the amount was significantly higher than basal levels of cells from the GEO group (Figure 3.8A). Upon activation with αCD3/αCD28 however, cells from HO individuals presented significantly higher levels of IL-17A when compared to both EN and GEO groups (Figure 3.8B). Furthermore, Th17 cells in each group were activated upon co-culture with αCD3/αCD28 compared to their unstimulated controls (Figure 3.8B). This pattern from cells of HO individuals was also reflected with IL-6 following both OvAg (Figure 3.8C) and αCD3/αCD28 (Figure 3.8D). In addition, background induction of IL-6 was higher in HO compared to GEO and EN (Figure 3.8C-D). As with IL-17A, no significant differences in the levels of IL-22 was seen (Figure 3.8E). However, TCR activation showed higher induction of IL-22 in each group compared to its control and also there was a significant increase of IL-22 in HO compared to both EN and GEO (Figure 3.8F). The secretion pattern of IL-1β following stimulation with OvAg (Figure 3.8G) or αCD3/αCD28 (Figure 3.8H) was similar to those of IL-22. Thus, although the overall OvAg-specific Th17-related responses were not highly significant in hyperreactive individuals, they did present a strong enhanced Th17 profile upon αCD3/αCD28 activation indicating a biased inflammatory profile.

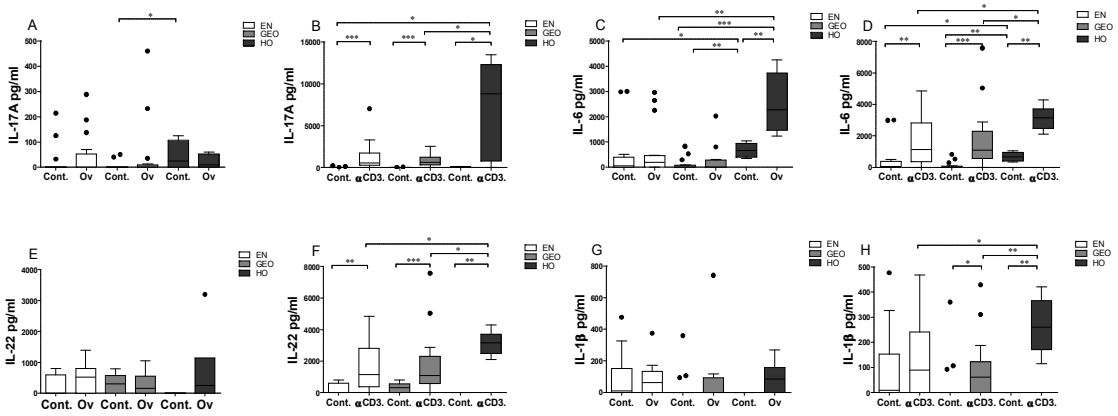

Figure 3.8: Elevated IL-17A and Th17-related cytokines in cell cultures from HO individuals following TCR activation.

Isolated PBMCs (1x10^5/well) from EN (n=16) and *O. volvulus*-infected GEO (n= 16) or HO (n=6) individuals were left either unstimulated or activated with αCD3/αCD28 (αCD3, 40,000beads/well) for 7 days. Thereafter, levels of IL-17A (A, B), IL-6 (C, D), IL-22 (E, F) and IL-1β (G, H) were measured in the culture supernatants using a FlowCytomix™ Multiplex kit via flow cytometry. Graphs show data as box whiskers with outliers. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001).

3.3.3. Increased Th9 OvAg-specific responses in hyperreactive onchocerciasis

Although the exact function of Th9 has yet to be completely determined, functions in immunity against helminth infections and/or the promotion of inflammatory diseases have been suggested [134]. Therefore, levels of Th9 were also investigated in human *Onchocerca volvulus*-infected patients. The secretion of IL-9 was significantly induced in cultures from HO individuals after OvAg stimulation when compared to either EN or GEO (Figure 3.9A). In addition, activation of PBMCs from EN with OvAg showed significantly increased amounts of IL-9 compared to GEO (Figure 3.9A). Moreover, whereas no detectable amounts were observed in GEO after OvAg activation, HO and EN exhibited increased IL-9 compared to controls (Figure 3.9A-B). However no significant differences in the induction of IL-9 was observed among the groups upon TCR activation with αCD3/αCD28 (Figure 3.9B) but there was an activation of Th9 cells by

αCD3/αCD28 stimulation in each group compared to its control (Figure 3.9B). This could suggest that Th9 cells are also implicated in the pathology of human onchocerciasis.

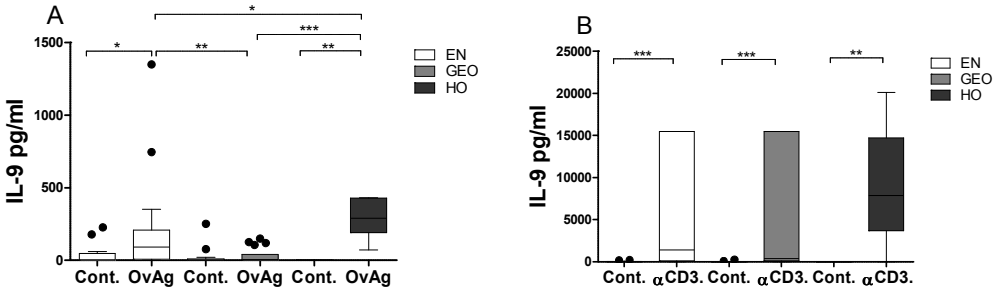

Figure 3.9: Strong induction of Ov-specific IL-9 secretion in cell cultures from hyperreactive individuals.

Isolated PBMCs (1x10^5/well) from EN (n=16) and *O. volvulus*-infected GEO (n= 16) or HO (n=6) individuals were left either unstimulated (Cont.) or activated with either an *O. volvulus* antigen extract (OvAg, 20µg/ml) or αCD3/αCD28 (αCD3, 40,000 beads/well) for 7 days. Thereafter, levels of IL-9 (A and B) were measured in the culture supernatants using a FlowCytomix™ Multiplex assay. Graphs show data as box whiskers tukey with outliers. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups (*p<0.05, **p<0.01, ***p<0.001).

3.3.4. Elevated antigen-specific TNF-α induction but not IL-12 and IL-2 in hyperreactive onchocerciasis

In figures 3.2A and 3.7E, the presented data shows an increased Th1 response in EN individuals by both flow cytometry and OvAg-stimulation. Expanding on those findings, levels of Th1-related cytokines, TNF-α, IL-2 and IL-12, were measured in cell cultures of PBMCs following activation with either OvAg or αCD3/αCD28. The results indicated an increase in TNF-α secretion in cell cultures from HO individuals compared to its control and to data from GEO groups (Figure 3.10A). The stimulation with αCD3/αCD28 induced a higher secretion of TNF-α in HO compared to both EN and GEO groups (Figure 3.10B). However, TNF-α levels were higher in EN, GEO and HO compared to their control (Figure 3.10B). Whereas IL-2 was not significantly induced by OvAg in cells from each group

(Figure 3.10C), TCR activation showed a significantly higher IL-2 secretion in HO compared to EN and GEO groups (Figure 3.10D). Low levels of IL-12p70 were detectable (Figure 3.10E-F). This suggests that OvAg-specific Th1 responsiveness in HO individuals is restricted to the production of TNF-α but not other Th1-related cytokines.

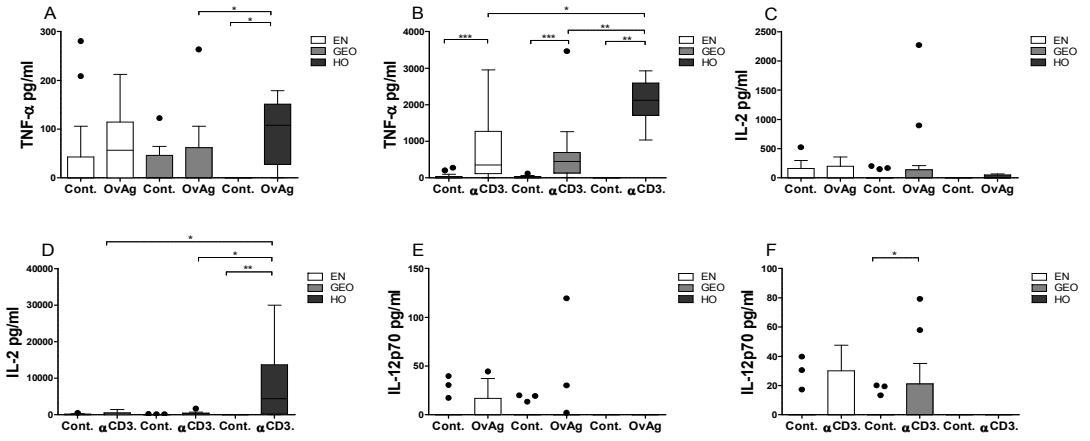

Figure 3.10: PBMCs from hyperreactive onchocerciasis individuals present elevated TNF-α secretion upon culture with Ov-antigen.

Isolated PBMCs (1x10^5/well) from EN (n=16) and *O. volvulus* -nfected GEO (n= 16) or HO (n=6) individuals were left either unstimulated (Cont.) or activated with either *O. volvulus* antigen extract (OvAg, 20µg/ml) or αCD3/αCD28 (αCD3, 40,000 beads/well) for 7 days. Thereafter, levels of TNF-α (A and B) and IL-2 (C-D) and IL-12p70 (E-F) were measured in the culture supernatants using a FlowCytomix™ Multiplex assay. Graphs show data as box whiskers with outliers. Asterisks show statistical differences (Kruskal-Wallis and Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001).

3.4. T helper cell profiling upon OvAg stimulation

In the previous section, the secretion of cytokines in the supernatants after PBMCs stimulation with antigen was investigated. The source of these cytokines could be a number of cells types within the PBMCs fraction. In addition, the cytokine profile from cell culture supernatants did not follow the trend observed after direct FACS analysis of cultured PBMCs. For instance HO individuals showed strong IL-17A responses after FACS but no differences were seen after OvAg stimulation. Therefore, we next characterized OvAg-stimulated cells per FACS analysis.

3.4.1. Higher antigen-specific $CD4^+T\text{-}bet^+$ but not $CD4^+IFN\text{-}\gamma^+$ T cells in HO individuals

In the above section (3.3.1.), peripheral blood mononuclear cells from EN showed elevated OvAg-specific IFN-γ secretion in the supernatants from EN compared to HO. To further investigate whether this IFN-γ induction was from Th1 cells, PBMCs from EN, GEO and HO were cultured alone (Cont.) or activated with either αCD3/αCD28 (αCD3.) or OvAg. After 7 days culture, cells were harvested and following PMA/Ionomycin activation, Th1 cell marker levels were determined by FACS analysis. Base line expression of T-bet in CD4 population was increased in HO compared to GEO and EN (Figure 3.11A-B). Furthermore, the HO group showed increased levels of $CD4^+T\text{-}bet^+$ T cells upon stimulation with OvAg (Figure 3.11A) and αCD3/αCD28 (Figure 3.11B) compared to other groups. However, no difference in $CD4^+IFN\text{-}\gamma^+$ T cells could be observed after co-culture with OvAg (Figure 3.11C). In contrast, TCR activation with αCD3/αCD28 (Figure 3.11D) showed increased expression of $IFN\text{-}\gamma^+$ CD4 T cells in GEO and HO compared to their non-stimulated control samples. The data did not confirm cytokine profiles from cell culture supernatants. This suggests that IFN-γ observed in cell culture supernatants may come from other cells in PBMCs.

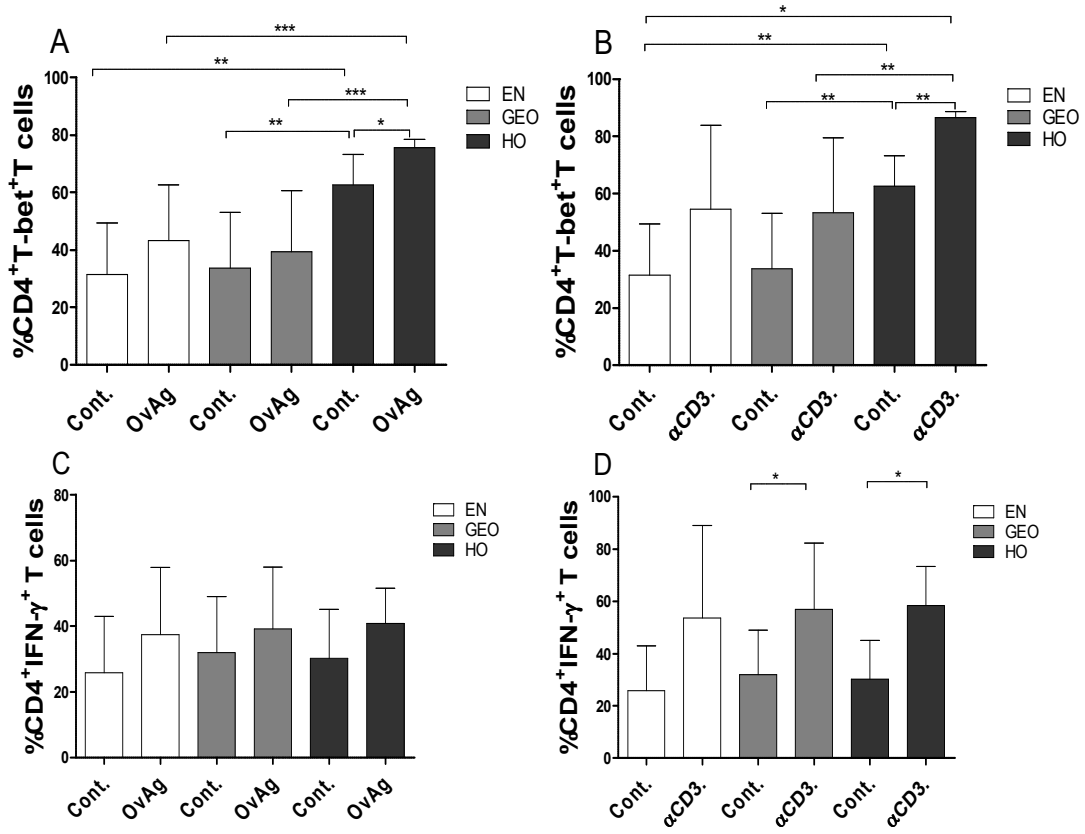

Figure 3.11: Elevated *Onchocerca volvulus*-specific CD4$^+$T-bet$^+$ but not CD4$^+$IFN-γ^+ cells in HO patients.

Peripheral blood mononuclear cells (1x10^5/well) from endemic normal EN(n=16), GEO(n=16) and HO(n=6) individuals were left unstimulated (Cont.) or stimulated with αCD3/αCD28 (αCD3. 40,000 beads/well) or *Onchocerca volvulus*-antigen (OvAg, 20µg/ml). After 7 days of culture, cells were harvested and stimulated with a cell stimulation cocktail containing PMA, Ionomycin, Brefeldin A and Monensin for 6 hours and then stained for the Th1 transcription factor T-bet and cytokine IFN-γ. Each dot represents the mean percentage± SD of CD4$^+$T-bet$^+$ and CD4$^+$IFN-γ^+ expression. Data of each group were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p< 0. 05, **p<0.01 and ***p<0.001.

3.4.2. *Onchocerca volvulus* adult worm antigen induces elevated GATA3 but not IL-4 expression in CD4$^+$ T cells from HO individuals

In section 3.2, it was demonstrated that hyperreactive onchocerciasis individuals exhibited an increase Th2 phenotype after FACS analysis and PCR array. This phenotype was specific for the parasite since upon OvAg activation, cell culture supernatants showed increased Th2 cytokines such as IL-5 and IL-13. Here it was aimed to investigate if the release of the these cytokines was from Th2 cells. Of

interest, the Th2 cytokine, IL-4 was not detectable in cell culture supernatants (data not shown). Therefore, cells from EN, GEO and HO were cultured either in medium alone (Cont.) or stimulated with either OvAg or αCD3/αCD28. After 7 days, cells were harvested and activated with PMA, Ionomycin, Brefeldin A and Monensin. After 6 hours, cells were characterized by FACS. The background expression of GATA3 was not different amongst the groups (Figure 3.12A-B). However, HO individuals exhibited increased $CD4^+GATA3^+$ T cells after OvAg stimulation compared to GEO and EN (Figure 3.12A).

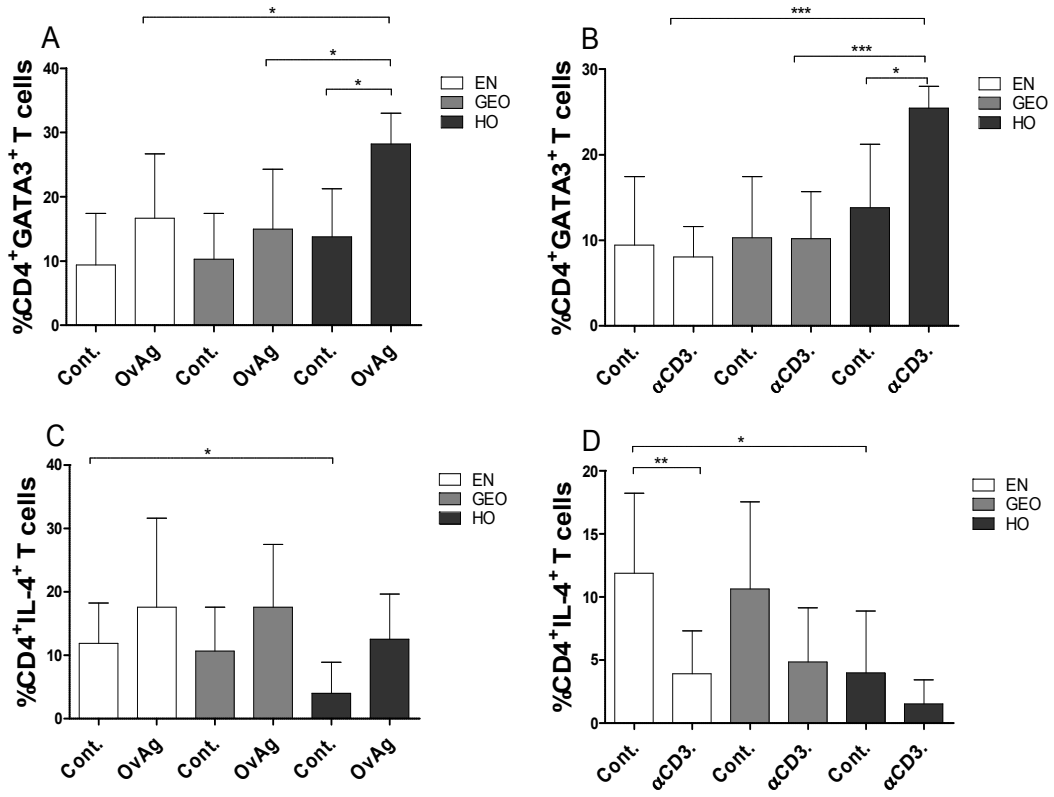

Figure 3.12: Elevated *Onchocerca volvulus*-specific $CD4^+GATA3^+$ but not $CD4^+IL\text{-}4^+$ T cells in HO individuals.

PBMCs($1x10^5$cell/well) from EN(n=16), GEO(n=16) and HO(n=6) individuals were left unstimulated (Cont.) or stimulated with αCD3/αCD28 (αCD3., 40,000 beads/well) or *Onchocerca volvulus*-antigen (OvAg, 20µg/ml). After 7 days of culture, cells were harvested and stimulated with a cell stimulation cocktail containing PMA, Ionomycin, Brefeldin A and Monensin for 6 hours .Thereafter cells were stained for the Th2 transcription factor GATA3 and cytokine IL-4. Each dot represents the mean percentage ± SD of $CD4^+GATA3^+$ (A and B) and $CD4^+IL\text{-}4^+$ (C and D) T cell populations. Data of each group were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p<0. 05 and ***p<0.001.

More interestingly, the expression of GATA3 in $CD4^+$ T cell populations did not differ from OvAg's trend after TCR activation with αCD3/αCD28 (Figure 3.12B). In contrast, background IL-4 expressing $CD4^+$ T cells was significantly lower in T cells from HO individuals when compared to EN and GEO groups (Figure 3.12C-D). No differences were observed in the levels of IL-4 amongst the groups upon OvAg activation (Figure 3.12C). Overall TCR activation decreased IL-4 expression in $CD4^+$ T cells compared to non-stimulated control samples and this decrease was significant in cultures from EN individuals (Figure 3.12D). This partially confirms our previous observations since HO individuals had OvAg-specific Th2 cytokines (increased IL-5 and IL-13) profiles in cell culture supernatants.

3.4.3. Elevated *Onchocerca volvulus*-specific $CD4^+RORC2^+$ but not $CD4^+IL\text{-}17^+$ cells in HO individuals

Strong Th17-related markers were observed after FACS analysis and PCR array in HO individuals (section 3.2). In contrast, IL-17A measured in cell culture supernatants was not different amongst the groups after OvAg stimulation and levels were also low. This could be that this cytokine was directly reabsorbed after its induction. Therefore, PBMCs from EN, GEO and HO were cultured for 7 days in medium alone (Cont.) or stimulated with either αCD3/αCD28 (αCD3) or OvAg. Thereafter, cells were harvested and activated with PMA, Ionomycin, Brefeldin A and Monensin. After 6 hours, T cells were characterized by FACS. As expected, OvAg and αCD3/αCD28 stimulation induced elevated $CD4^+RORC2^+$ T cells in HO individuals compared to EN and GEO persons (Figure 3.13A-B). Interestingly, background expression of RORC2 in $CD4^+$ T cell populations was higher in HO compared to EN and GEO (Figure 3.13A-B). TCR activation using αCD3/αCD28 induced increased expression of $CD4^+RORC2^+$ T cells in EN and HO compared to

controls (Figure 3.13B). In contrast, the trend of $CD4^+IL\text{-}17A^+$ T cells did not correlate with RORC2 expression neither with OvAg stimulation (Figure 3.13C) nor TCR activation (Figure 3.13D). Basal expression of IL-17A was higher in EN and GEO compared to HO individuals (Figure 3.13C-D). Interestingly, OvAg stimulation induced IL-17A secretion in GEO compared to HO groups (Figure 3.13C). This data could indicate a negative feedback control of IL-17A induction and since cells were pre-stimulated with PMA, this could boost RORC2 expression but not the release of IL-17A.

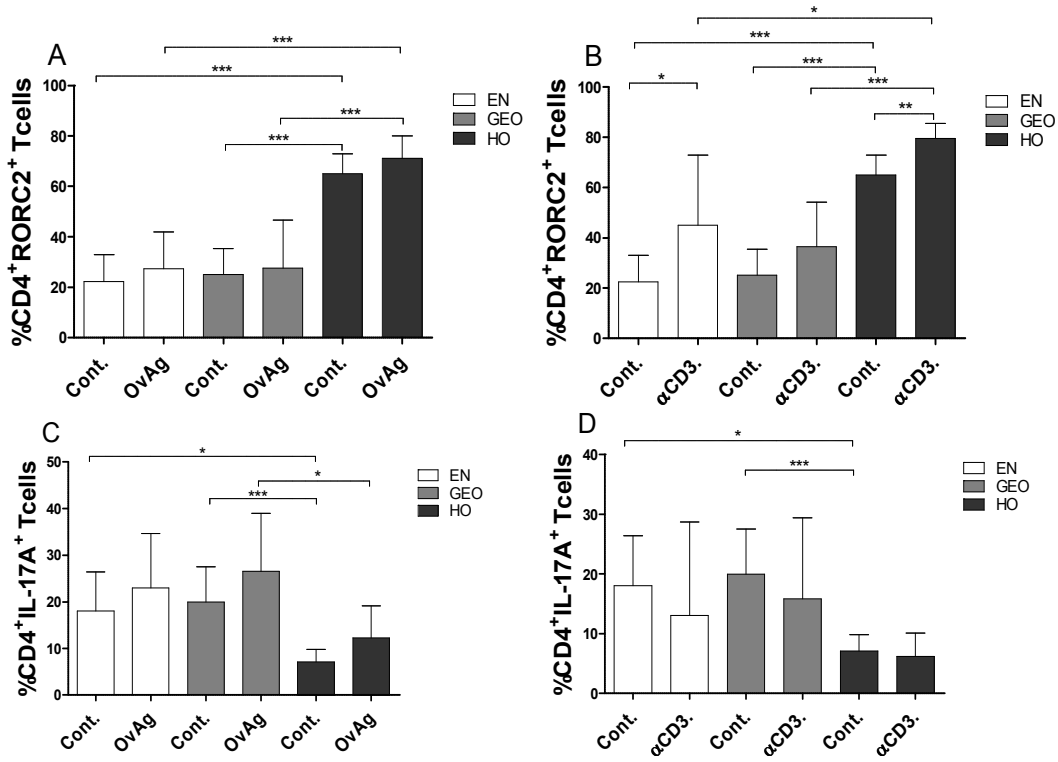

Figure 3.13: Elevated *Onchocerca volvulus*-specific $CD4^+RORC2^+$ but not $CD4^+IL\text{-}17A^+$ T cells in HO individuals.

PBMCs($1x10^5$cell/well) from EN(n=16), GEO(n=16) and HO(n=6) individuals were left unstimulated (Cont.) or stimulated with OvAg (20μg/ml) or αCD3/αCD28(αCD3.,40,000 beads/well). After 7 days culture, cells were harvested and stimulated with PMA, Ionomycin, Brefeldin A and Monensin for 6 hours and were stained for the Th17 transcription factor RORC2 and cytokine IL-17A. Each dot represents the mean percentage ± SD of $CD4^+RORC2^+$ and $CD4^+IL\text{-}17A^+$ T cell frequency. Data of each group were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p< 0. 05 and ***p<0.001.

3.4.4. Increased *Onchocerca volvulus*-specific $CD4^+IL\text{-}10^+$ T cells in PBMCs cultures of HO individuals

$CD4^+$ T cells were shown to be the predominant IL-10 producing cells in filarial-infected patients [114]. In cell culture supernatant, IL-10 was only observed at very low levels. (Figure 3.7H). In order to assess the expression of IL-10 and Foxp3 in the $CD4^+$ T population, cells from EN, GEO and HO were cultured and left alone (Cont.) or stimulated with either αCD3/αCD28 (αCD3) or OvAg. After 7 days, cells were harvested and activated with PMA, Ionomycin, Brefeldin A and Monensin and 6 hours later, cells were stained and characterized by FACS. The OvAg stimulation slightly increased the expression of Foxp3 in $CD4^+$ T cell populations compared to the unstimulated controls and this was significantly different in HO and its control (Figure 3.14A). No differences in expression of Foxp3 was observed among the groups before stimulation (Figure 3.14A) or after activation with either OvAg or αCD3/αCD28 (Figure 3.14B). Interestingly, in unstimulated cells from HO individuals, IL-10-producing $CD4^+$ T cells were significantly elevated compared to other controls (Figure 3.14C). This was again significantly elevated in HO compared to EN and GEO after OvAg stimulation (Figure 3.14C). This phenotype did not changed upon TCR activation with αCD3/αCD28 (Figure 3.14D).

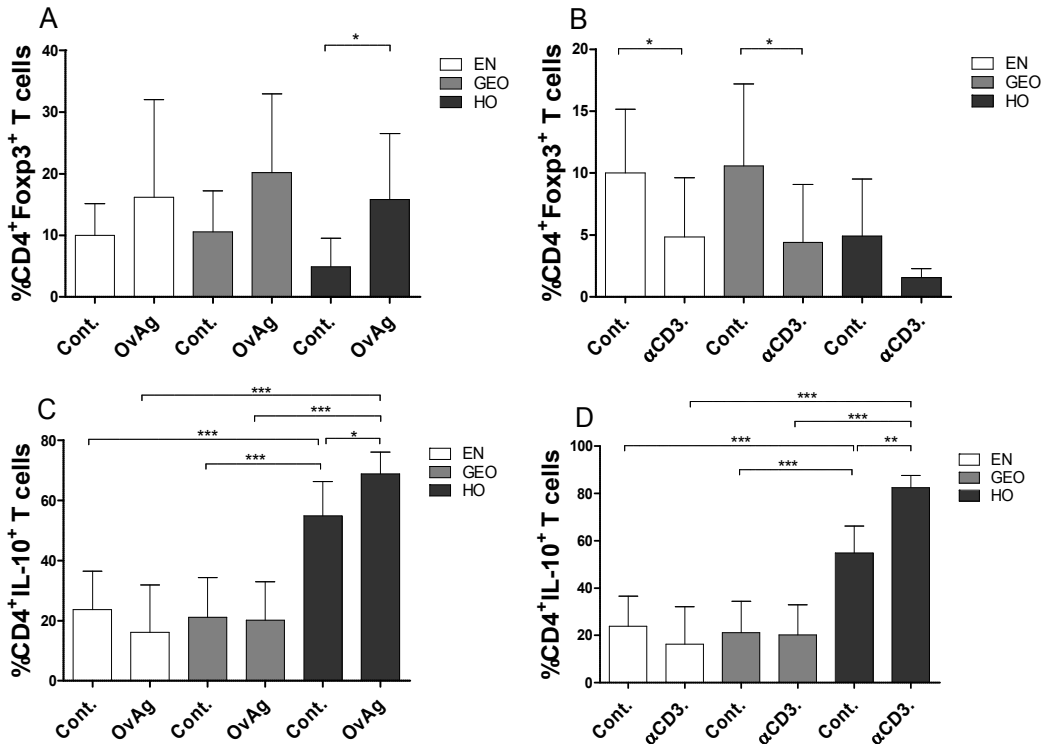

Figure 3.14: Increased *Onchocerca volvulus*-specific $CD4^+IL\text{-}10^+$ T cells in HO individuals.

PBMCs from EN, GEO and HO individuals were left unstimulated (Cont.) or stimulated with OvAg (20μg/ml) or αCD3/αCD28 (αCD3.,40,000 beads/well). After 7 days culture, cells were harvested and stimulated with PMA, Ionomycin, Brefeldin A and Monensin for 6 hours and were stained for Foxp3 and IL-10. Each dot represents the mean percentage ± SD of $CD4^+Foxp3^+$ (A, B) and $CD4^+IL\text{-}10^+$ (C,D) expression. Data of each group were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p< 0. 05, **p< 0.01 and ***p<0.001.

3.5. Antibody profiles in *Onchocerca volvulus* infected individuals

The findings in section 3.2.1, showed that the frequencies of B cells was not different between EN, GEO and HO but memory T helper cells were elevated in HO individuals compared to the other groups. Further investigations highlighted the predominant Th2 and Th17 responses in HO patients. In this section, the role of B cell responses in hyperreactive onchocerciasis has been delineated using *in vitro* cell culture assays.

3.5.1. Elevated OvAg-specific IgG2, IgA and IgM in hyperreactive onchocerciasis

In order to investigate filarial-specific IgG1, IgG2, IgG3, IgA and IgM antibody responses in HO individuals, PBMCs from *Onchocerca volvulus*-infected individuals (GEO and HO) and EN were left unstimulated (Cont.) or stimulated with either αCD3/αCD28 mAb microbeads (αCD3) or OvAg. Using a human Immunoglobulin Isotyping 6plex, levels of IgG1 and IgG2, IgG3, IgA and IgM were measured.

There was no significant differences in the amounts of IgG1, IgG2 and IgG3 secreted in the cell culture supernatants in any group either with or without stimulation (Figure 3.15A-F). Although levels of IgG3 were increased in EN and GEO individuals compared to the HO group upon activation with OvAg these levels were not significant (Figure 3.15A). The secretion of IgG2 was elevated in cultures from HO individuals compared to those from either EN or GEO groups after activation with OvAg (Figure 3.15D) and αCD3/αCD28 (Figure 3.15D). This data suggests that while OvAg-specific IgG2 induction is associated with hyperreactivity, IgG3 seems to be linked to protection in GEO and to putative immunity in EN.

Regarding the secretion of IgA by activated B cells, the data showed a significant increase in IgA production in cultures from the HO group compared to EN and GEO upon OvAg stimulation (Figure 3.15G). IgA was also induced by the Ov antigen in cultures from EN individuals when compared to unstimulated controsl (Figure 3.15G and H). This was also the case upon T cell activation by αCD3/αCD28 since elevated amounts of IgA was observed in cultures from EN when compared to unstimulated controls (Figure 3.15H). Moreover, levels of IgA

were significantly increased in cultures from EN individuals when compared to either GEO or HO groups (Figure 3.15H). Interestingly, OvAg-stimulated B cells from HO to produced elevated amounts of IgM compared to its control and to activated cultures from EN and GEO groups (Figure 3.15I). This trend was similar after TCR activation (Figure 3.15G). These results indicate that IgA and IgM stimulated B cells may be involved in the disease pathogenesis of onchocerciasis.

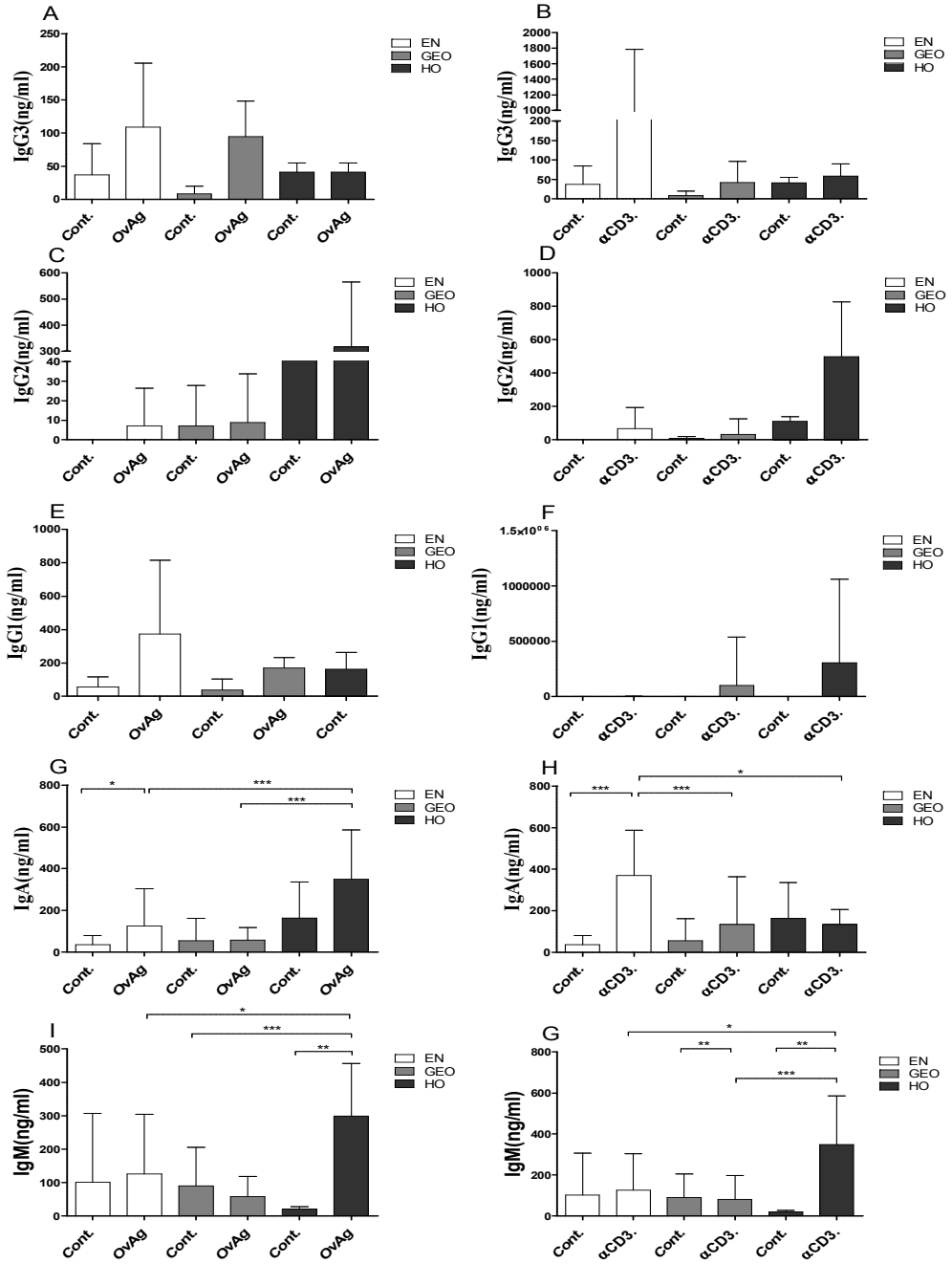

Figure 3.15: ***Onchocerca volvulus*** **antigen-specific antibodies induced in HO individuals**.

Peripheral blood mononuclear cells (PBMCs, 1x10^{5}cell/well) from Endemic normal (EN, n=16), generalized onchocerciasis (GEO, n=16) and hyperreactive onchocerciasis (HO, n=6) individuals were thawed and stimulated either with *Onchocerca volvulus*-antigen (OvAg, 20μg/ml) or αCD3/αCD28 (αCD3, 40,000 beads/well). After 14 days culture, supernatants were taken and levels of IgG1, IgG2, IgG3, IgA and IgM secretion were measured using a human Immunoglobulin Isotyping 6plex assay. Graphs show box whiskers with median, interquartile range and outliers. Data of each group were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p<0, 05, **p<0, 01 and ***p<0,001.

3.5.2. Elevated IgE responses in hyperreactive onchocerciasis individuals

In human filariasis, while IgG4 has been shown to be associated with immunomodulation, IgE has been linked to the development of pathology [6]. To assess the association of IgG4 and IgE in different clinical manifestations in human onchocerciasis, PBMCs from GEO, HO and EN were left unstimulated (Cont.) or stimulated with either αCD3/αCD28 (αCD3) or OvAg. After 14 days culture, supernatants were harvested and the induction of IgG4 and IgE were measured using Flowcytomix multiplex systems. While spontaneous release of IgG4 is higher in GEO compared to EN and HO (Figure 3.16A), OvAg stimulation induced significant increases of IgG4 in EN and GEO compared to their unstimulated controls (Figure 3.16A). No differences however were observed upon activation with αCD3/αCD28 between the three groups (Figure 3.16B). In contrast, basal secretion of IgE was increased in HO compared to GEO and HO (Figure 3.16C). OvAg did not induce IgE production in HO but levels were increased in EN and GEO compared to controls (Figure 3.16C). Moreover, αCD3/αCD28 stimulation did induce higher levels of IgE in cultures from HO and EN individuals when compared to unstimulated controls but no differences were observed amongst the groups (Figure 3.16D). The ratio IgE vs IgG4 was elevated in stimulated and non-stimulated PBMCs from HO compared to EN (Figure 3.16E-F). In concordance with the literature, these results indicate that IgE could be linked to disease manifestation while IgG4 is associated with protection in GEO individuals.

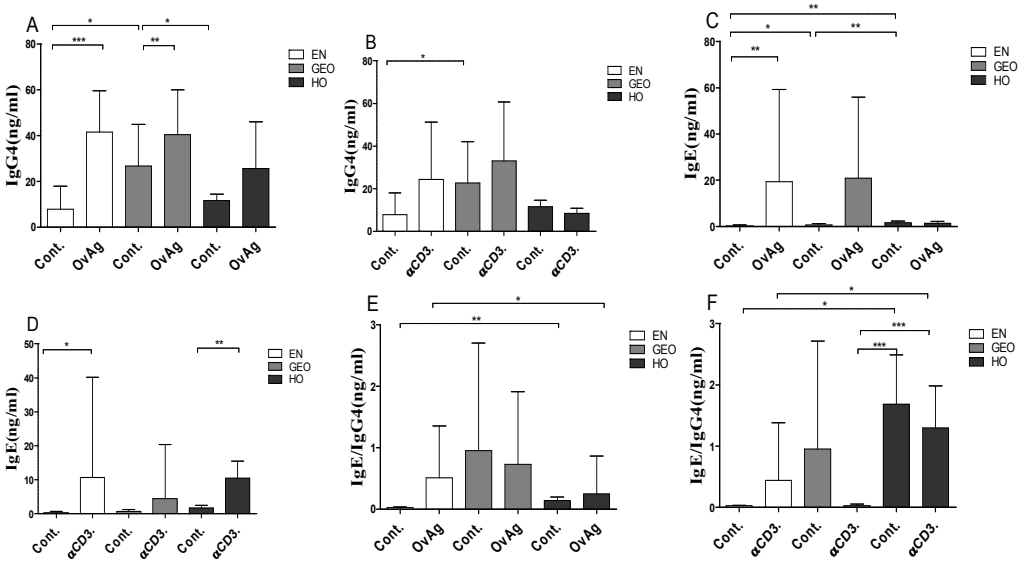

Figure 3.16: Elevated basal IgE and IgG4 levels in cell cultures from HO and GEO individuals respectively.

PBMCs ($1x10^5$cell/ml) from EN (n=16), GEO (n=16) and HO (n=6) individuals were left alone (cont.) or stimulated either with *Onchocerca volvulus*-antigen (OvAg, 20μg/ml) or αCD3/αCD28(αCD3, 40,000beads/well). After 14 days culture, supernatants were taken and secreted levels of IgG4 and IgE were measured by FlowCytomix Multiplex assays. Graphs show box whiskers with median, interquartile range and outliers. Data of each group were compared using Kruskal-Wallis and Mann-Whitney test and significant differences are given as *p<0, 05, **p<0, 01 and ***p<0,001.

3.6. *Onchocerca volvulus* modulates Th17 cell generation in vitro

In the previous sections, increased Th17 responses were associated with HO individuals compared to EN and GEO. Considering the characteristics of GEO compared to HO individuals (Table 1), GEO patients presented elevated numbers of nodules (implying higher worm load too) compared to HO who have little or no nodules. A possible scenario that could explain the differences in the levels of Th17 between both groups may be the modulation of immune response in GEO individuals by the adult worm. Indeed, helminth-derived antigens were shown to have a strong inhibitory effect on T helper cell commitment [8]. Therefore, using healthy donors, the modulation of Th17 responses by OvAg was studied *in vitro*.

3.6.1. IL-6 in combination with IL-1β induced Th17 cells polarization

Th17 cells are committed in the presence of low levels of TGF-β and higher amounts of IL-6 in combination with IL-1β and IL-23 [135]. In order to generate IL-17A producing $CD4^+$ T cells *in vitro*, PBMCs were either left alone or stimulated with αCD3/αCD28 in the presence or absence of a chemokine cocktail (CC) containing IL-6 and IL-1β.

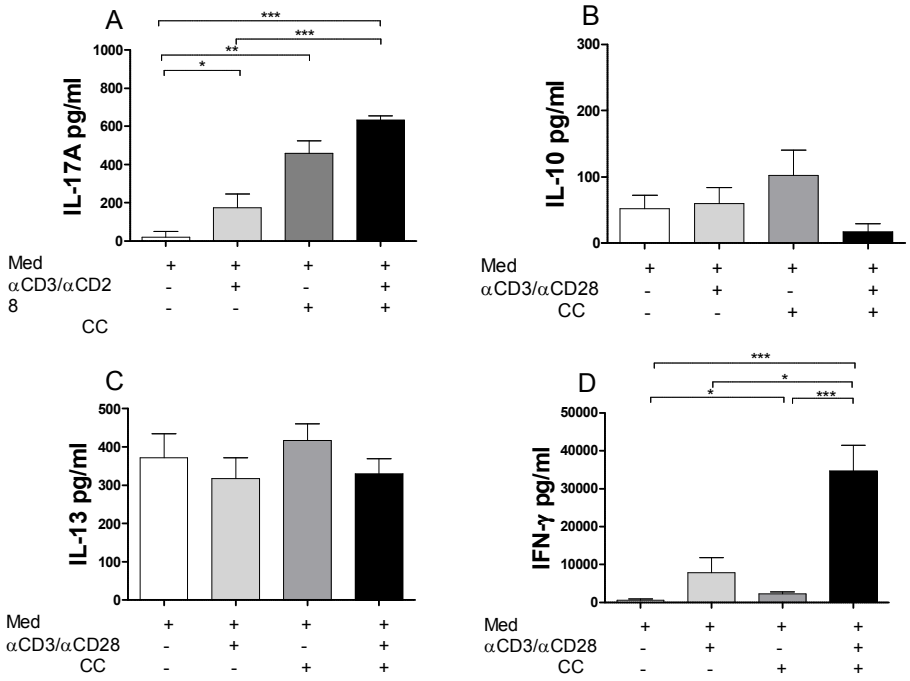

Figure 3.17: IL-6 in combination with IL-1β induces higher secretions of IL-17A.

PBMCs($1x10^5$cells/ml) from European healthy donors (n=12) were left alone (Med) or stimulated with αCD3/αCD28 (20,000 beads/well) in the presence or absence of chemokine cocktail containing IL-6 and IL-1β (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml). After 7 days culture, supernatants were taken and levels of IL-17A (A), IL-10 (B), IL-13 (C) and IFN-γ (D) were measured using ELISA. Bars represent mean ± SD of each cytokine level. Asterisks show statistical differences (Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p<0,001).

The presence of the chemokine cocktail significantly induced the secretion of IL-17A (Figure 3.17A). In contrast, no induction of either IL-10 (Figure 3.17B) or IL-13 (Figure 3.17C) could be observed. Indeed, the combination of CC and

αCD3/αCD28 reduced IL-10 levels (Figure 3.17B). Moreover, these CC could also provoke significant release of IFN-γ (Figure 7D). This indicates that TCR activation in the presence of IL-6 and IL-1β could lead to the generation of Th17 cells and Th1 cells.

3.6.2. *Onchocerca volvulus* adult antigen inhibits the induction of IL-17A by IL-6 and IL-1β

To assess the immunomodulatory capacity of OvAg on Th17 cells development, PBMCs were either left unstimulated or activated with αCD3/αCD28 in an IL-6 and IL-1β milieu in the presence or absence of OvAg. Following 7 days culture, levels of IL-17A, IL-10, IL-13 and IFN-γ were measured. The addition of OvAg significantly down-regulated the secretion of both IL-17A (Figure 3.18A) and IL-13 (Figure 3.18B) in the presence of IL-6 and IL-1β. In contrast, OvAg increased the induction of either IL-10 (Figure 3.18C) or IFN-γ (Figure 3.18D) compared to those without OvAg stimulation. These data suggest that OvAg could modulate or inhibit Th2 and Th17 responses.

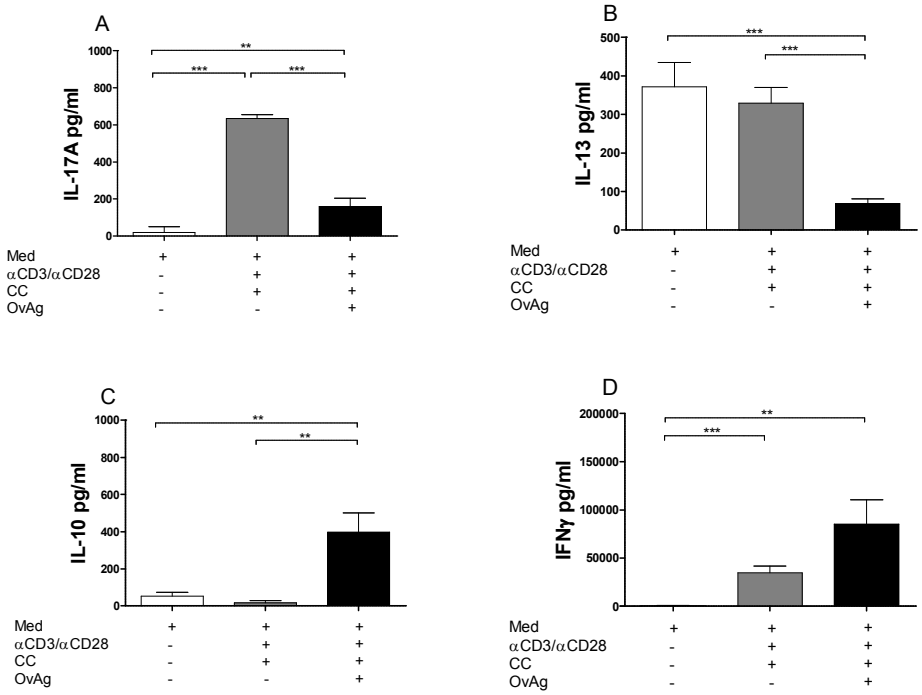

Figure 3.18: *Onchocerca volvulus* adult antigen inhibits the induction of IL-17A.

PBMCs (1x10^{5}cells/ml) from European healthy donors (n=12) were left alone (Med) or stimulated with **α**CD3/**α**CD28 (20,000 beads/well) in a chemokine cocktail milieu containing IL-6 and IL-1β (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml) in presence or absence of OvAg (12.5□in p). After 7 days of culture, supernatants were removed and the induction of IL-17A (A), IL-13 (B), IL-10 (C) and IFN-γ (D) were measured using ELISA. Bars represent mean ± SD of each cytokines level. Asterisks show statistical differences (Mann Whitney test) between the groups indicated by the brackets (**p<0.01 and ***p<0,001).

3.6.3. Th2 and IL-10 cytokines reduces the secretion levels of IL-17A

To investigate whether OvAg used Th2 or IL-10 pathways to inhibit the induction of IL-17A by IL-6 and IL-1β, PBMCs were either left unstimulated or activated with αCD3/αCD28 in the CC milieu and in addition, in the presence or absence of rhIL-13, rhIL-4, rhIL-10 or with the combination of rhIL-4 and rhIL-13. The addition of each cytokine (Figure 3.19A, B and C) or a combination of IL-4 and IL-13 (Figure 3.19D) significantly reduced the secretion of IL-17A suggesting that OvAg was modulating Th17 cell development using Th2 and/or IL-10 cytokine pathways.

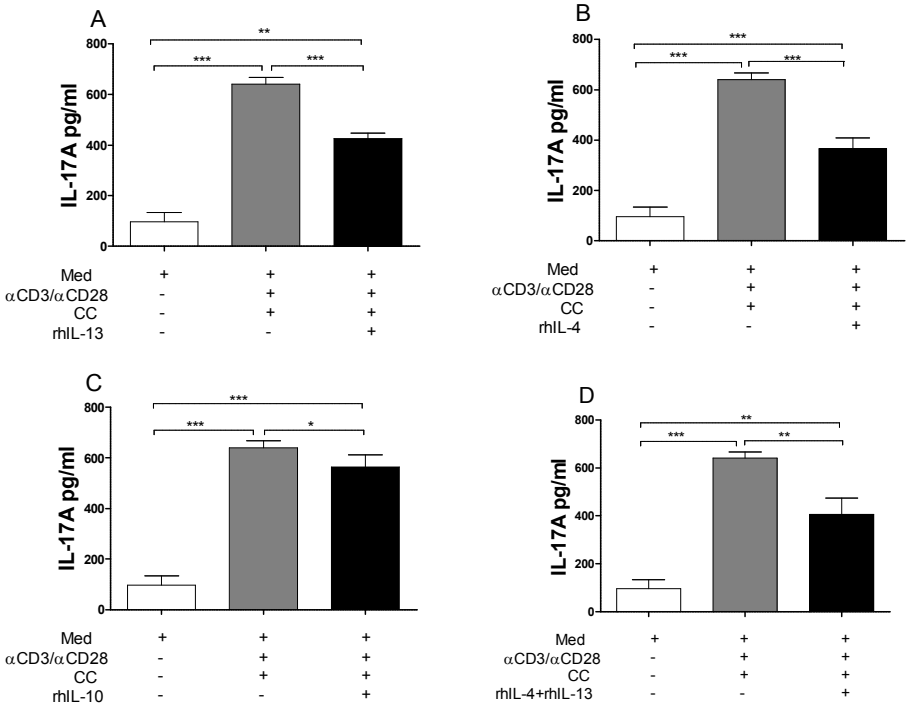

Figure 3.19: Th2 or IL-10 cytokines reduce the secretion levels of IL-17A.

$1x10^5$ cells/ml of PBMCs from European healthy donors (n=12) were left alone (Med) or stimulated with αCD3/αCD28 (20,000 beads/well) in a chemokine cocktail milieu containing IL-6 and IL-1β (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml) in the presence or absence of either (A) rhIL-13 (10ng/ml), (B) rhIL-4(10ng/ml), (C) rhIL-10(10ng/ml) or (D) with the combination of rhIL-4 and rhIL-13. After 7 days culture, supernatants were removed and the induction of IL-17A was measured using ELISA. Bars represent mean ± SD of IL-17A levels. Asterisks show statistical differences (Mann Whitney test) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p<0,001).

3.6.4. Neutralizing Th2 related cytokines and IL-10 did not abolish the suppressive effect of OvAg on Th17 cells development

In order to confirm the hypothesis that OvAg could use either Th2 and/or IL-10 cytokine pathways to modulate Th17 cell development, PBMCs were either left unstimulated or activated with αCD3/αCD28 in an IL-6 and IL-1β milieu in the presence or absence of OvAg and neutralization antibodies against IL-13, IL-4 and IL-10 or respective isotype control. Blocking these cytokines (Figure 3.20A, B and C) or blocking them all simultaneously (Figure 3.20D) did not abolish the

suppressive effect of OvAg. This indicates that OvAg is using another pathway to modulate the development of Th17 cells.

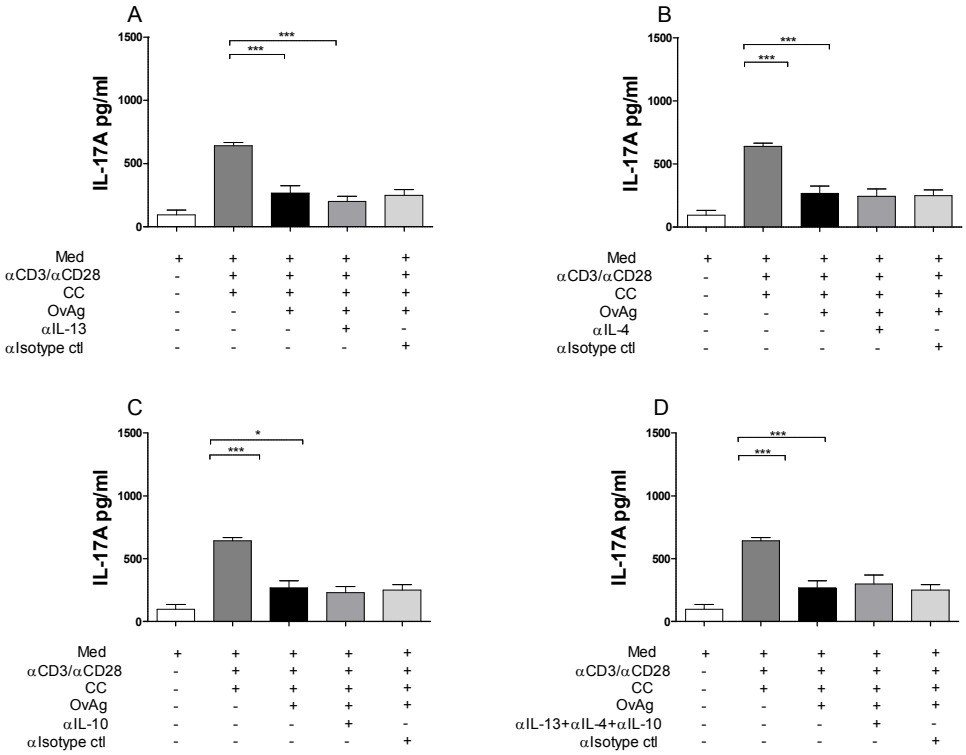

Figure 3.20: Neutralizing Th2 related cytokines or IL-10 does not affect OvAg mediated suppressing of IL-17A production.

$1x10^5$ PBMCs/ml from European healthy donors (n=12) were left alone (Med) or stimulated with αCD3/αCD28 (20,000 beads/well) in a chemokine cocktail milieu containing IL-6 and IL-1β (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml) in the presence or absence of OvAg (12.5μg/ml) and neutralization antibodies against (A) IL-13 (αIL-13, 1μg/ml), (B) IL-4 (αIL-4, 1μg/ml), (C) IL-10 (αIL-10, 1μg/ml) and (D) with the combination of αIL-13, αIL-4 and αIL-10. Cultures were also stimulated in the presence of respective isotype controls. After 7 days culture, supernatants were removed and secretion of IL-17A was measured by ELISA. Bars represent mean ± SD of IL-17A levels. Asterisks show statistical differences (Mann Whitney test) between the groups indicated by the brackets (*p<0.05, and ***p<0,001).

3.6.5. Indirect suppression of IL-17A secretion via monocytes but not B cells

To assess at the cellular level the modulatory effect of OvAg on IL-17A production, PBMCs, T cells alone and co-cultures of T cells with either B cells or monocytes were either left unstimulated or activated with αCD3/αCD28 in an IL-6 and IL-1β milieu in the presence or absence of OvAg.

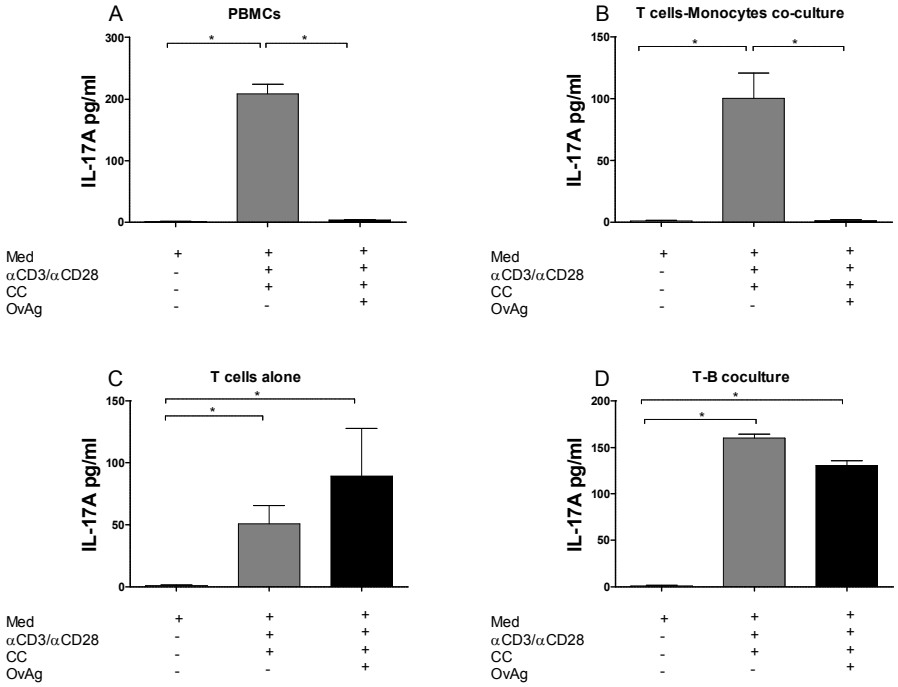

Figure 3.21: OvAg reduce IL-17A secretion by modulating monocytes.

(A) PBMCs (1x10^{5}cells/ml), (B) co-culture T cells (1x10^{5}cells/ml) and Monocytes (1x10^{5}cells/ml), (C) T cells alone(1x10^{5}cells/ml) and (D) co-culture of T cells and B cell (1x10^{5}cells/ml) were either left unstimulated or activated with αCD3/αCD28 (20,000 beads/well) in a chemokine cocktail milieu containing IL-6 and IL-1β (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml) in the presence or absence of OvAg (12.5μg/ml). After 7 days of culture, supernatants were screened for the secretion of IL-17A by ELISA. Bars represent mean ± SD of IL-17A levels. Asterisks show statistical differences (Mann Whitney test) between the groups indicated by the brackets (*p<0.05).

The suppressive trend of IL-17A observed with PBMCs after stimulation with OvAg (Figure 3.21A) was similar in co-cultures of T cell-monocytes (Figure 3.21B). In contrast, OvAg could not reduce IL-17A secretion with T cells alone or co-cultured with B cells (Figure 3.21C and D respectively). This indicates that OvAg did not interfere directly with T cells but acted on monocytes.

3.7. Transformation of the GEO phenotype into a HO phenotype

The mechanism underlining the development of HO phenotype in some individuals remains unclear. It was shown that Treg under inflammatory conditions could switch to Th17 responses [136-138]. Since the data showed that GEO individuals

have an elevated regulatory phenotype, in this section the following experiments describe potential pathways which could transform a regulatory phenotype into a Th17 one in vitro by activating innate receptors.

3.7.1. Increased pro-inflammatory cytokine secretion in cultures from GEO individuals after innate receptor stimulation

The mechanisms underlining the differences observed between GEO and HO individuals is still not clearly defined. In section 3.1 and 3.2 of this study, it was shown that HO individuals, those with low MF load, exhibited strong pro-inflammatory Th17 responses while GEO individuals, that is those with high MF load and adults worms, showed a strong regulatory phenotype. Therefore, PBMCs from EN and GEO individuals were stimulated with C12-iE-DAP (a PGN-like molecule, 100ng/ml) which is a NOD1 ligand, ODN 2006 (TLR9 ligand, 1μM), LPS (TLR4 ligand, 10ng/ml) and Muramyl dipeptide (10μg/ml), a NOD2 ligand for 7 days to find out whether additional stimulation of these receptors could switch the GEO phenotype to a hyperreactive one. Interestingly, TLR4 stimulation down-regulated both IL-17A and IFN-γ induction in cultures from GEO individuals (Figure 3.22A and Figure 3.23A respectively). The data show that whilst NOD1L stimulation significantly increased IL-17A and IFN-γ production in GEO patients compared to EN (Figure 3.22B and Figure 3.23B respectively). Upon NOD2 activation no effect was observed regarding IL-17A or IFN-γ secretion in cultures from GEO individuals when compared to EN (Figure 3.22C and Figure 3.23C respectively). TLR9 activation decreased IL-17A but not IFN-γ production in GEO cultures compared to EN (Figure 3.22D and Figure 3.23D respectively). Moreover, the activation of either TLR4, NOD1 or NOD2 did not induce IL-4 secretion in either EN or GEO (Figure 3.24A, B and C). IL-4 induction was significantly

decreased in EN after TLR9 stimulation compared to control well but an increased level of this cytokine was observed in cultures from GEO compared to those from EN (Figure 3.24D). Regarding IL-10 secretion, no differences were observed after TLR4 activation (Figure 3.25A). An increase induction was seen in GEO compared to EN upon NOD1 and NOD2 stimulation (Figure 3.25B and C). TLR9-activated PBMCs did not induce any differential secretion amongst the groups (Figure 3.25D). Overall, these data indicated that additional TLR4, TLR9 and NOD1 but not NOD2 stimulation could switch GEO phenotype to HO.

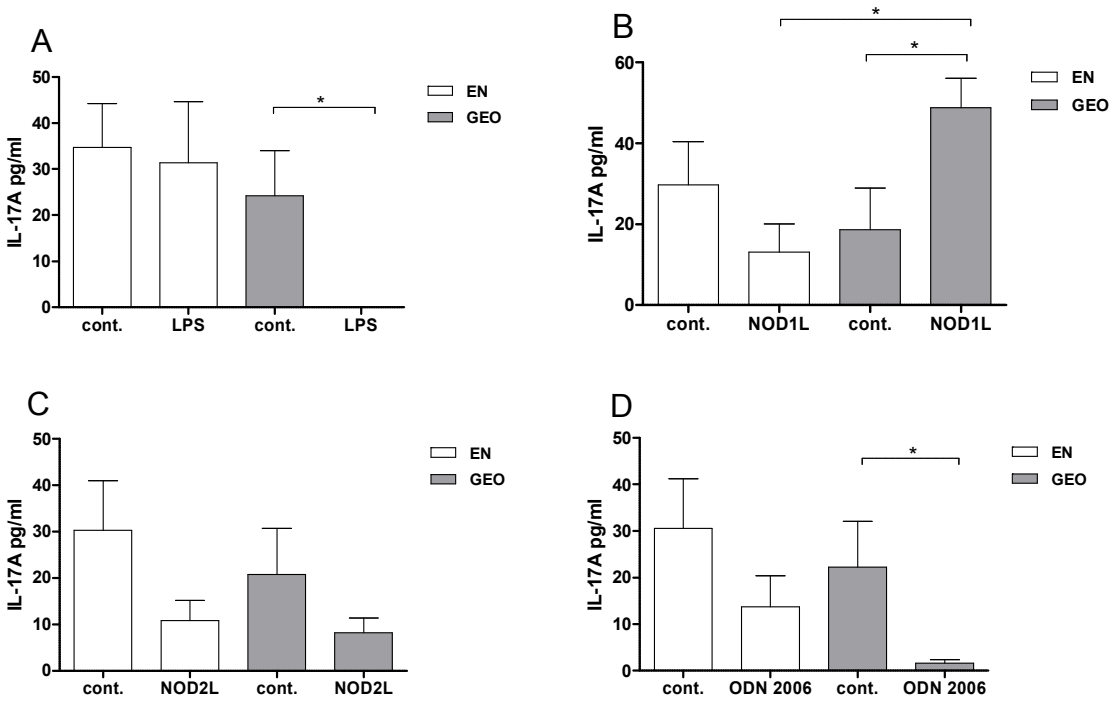

Figure 3.22: Increased IL-17A in GEO after NOD1 stimulation.

PBMCs ($1x10^5$cells/ml) from EN (n=7) and GEO (n=7) individuals were stimulated with (A) C12-iE-DAP (PGN-like molecule, 100ng/ml) a NOD1 ligand, (B) ODN 2006 (TLR9 ligand, 1μM), (C) LPS (TLR4 ligand, 10ng/ml) and (D) Muramyl dipeptide (10μg/ml) a NOD2 ligand for 7 days and the secretion of IL-17A was measured by a Flowcytomix multiplex assay. Bars represent the mean ± SEM of IL-17A induction. Statistics were performed using 1way ANOVA. Asterisks show statistical differences between the groups indicated by the brackets (*p<0.05).

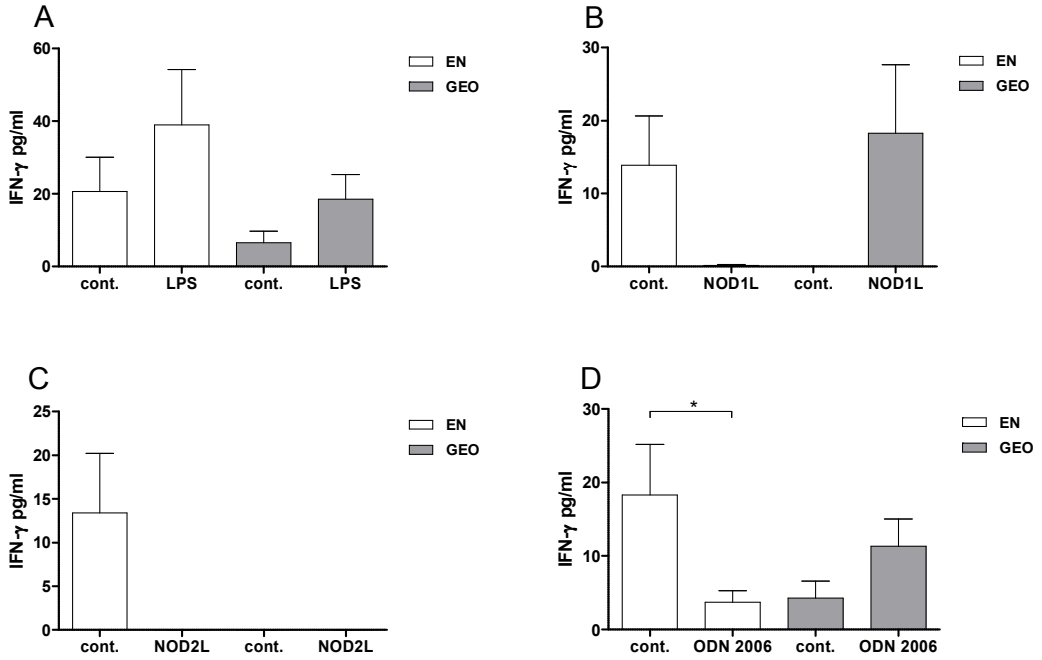

Figure 3.23: High IFN-γ in GEO upon NOD1, TLR9 and TLR4 stimulation.

PBMCs($1x10^5$cells/ml) from EN (n=7) and GEO (n=7) individuals were activated with (A) C12-iE-DAP (PGN-like molecule, 100ng/ml) a NOD1 ligand, (B) ODN 2006 (TLR9 ligand, 1μM), (C) LPS (TLR4 ligand, 10ng/ml) and (D) Muramyl dipeptide (10μg/ml) a NOD2 ligand for 7 days and the secretion of IFN.γ was measured using Flowcytomix multiplex assay. Bars represent the mean ± SEM of IFN.γ induction. Statistics were performed using 1way ANOVA. Asterisks show statistical differences between the groups indicated by the brackets (*p<0.05).

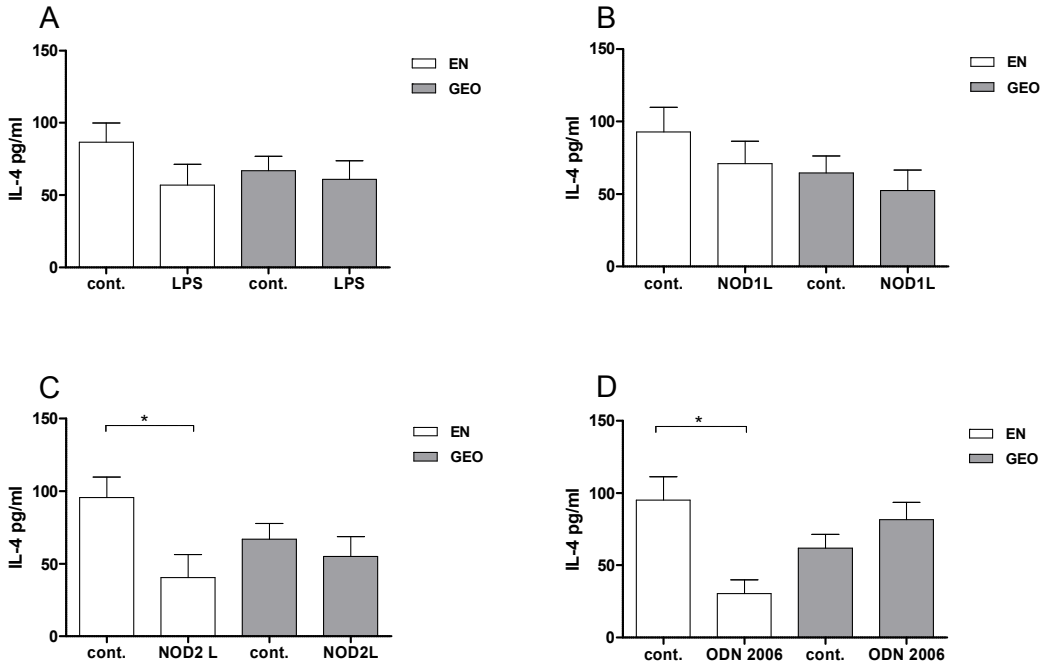

Figure 3.24: Increased IL-4 in GEO after NOD2 and TLR9 activation.

Cells ($1x10^5$ cells/ml) from EN (n=7) and GEO (n=7) individuals were stimulated with (A) C12-iE-DAP (PGN-like molecule, 100ng/ml) a NOD1 ligand, (B) ODN 2006 (TLR9 ligand, 1μM), (C) LPS (TLR4 ligand, 10ng/ml) and (D) Muramyl dipeptide (10μg/ml) a NOD2 ligand for 7 days and the secretion of IL-4 was measured by Flowcytomix. Bars represent the mean ± SEM of IL-4 induction. Statistics were performed using 1 way ANOVA. Asterisks show statistical differences between the groups indicated by the brackets (*$p<0.05$).

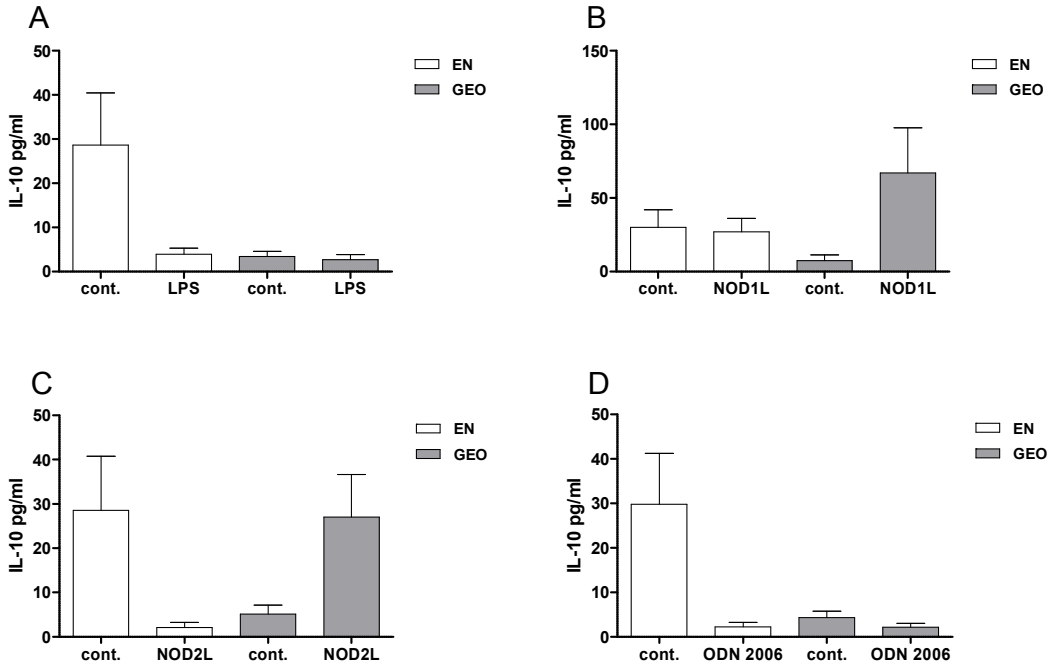

Figure 3.25: Augmented IL-10 in GEO upon NOD1 and NOD2 stimulation. PBMCs(1x10^{5}cells/ml) from EN (n=7) and GEO (n=7) individuals were stimulated with (A) C12-iE-DAP (PGN-like molecule, 100ng/ml) a NOD1 ligand, (B) ODN 2006 (TLR9 ligand, 1μM), (C) LPS (TLR4 ligand, 10ng/ml) and (D) Muramyl dipeptide (10μg/ml) a NOD2 ligand for 7 days and the secretion of IL-10 was measured by Flowcytomix. Bars represent the mean $\pm$ SEM of IL-10 induction. Statistics were performed using 1way ANOVA.

3.7.2. IL-6 and IL-1β induced IL-17A and IFN-γ in GEO and EN respectively

In section 3.8, the triggering of NOD and TLRs elicited the induction of IL-17A production in PBMCs from GEO individuals. In order to assess the mechanism by which additional TLR4, TLR9 and NOD1 stimulation induced the secretion of IL-17A and IFN-γ in cell cultures from GEO individuals, cells from EN and GEO were activated with αCD3/αCD28 in the presence of IL-6 and IL-1β. After 7 days of culture, IL-17A, IFN-γ, IL-4 and IL-10 were measured from cell culture supernatants. Significant increases of IL-17A and IFN-γ were observed in cultures from EN and GEO groups when comparing unstimulated controls with those with TCR activation in the presence of IL-6 and IL-1β (Figure 3.26A and B). The secretion of IL-17A was higher in cultures from GEO individuals when compared to

EN (Figure 3.26A). This was not the case for IFN-γ since levels were increased in EN compared to GEO group (Figure 3.26B). No differences were observed regarding IL-4 secretion (Figure 3.26C) but IL-10 was higher in EN cultures compared to GEO after addition of IL-6 and IL-1β (Figure 3.26D). These findings could suggest that TLRs and NLRs activation induce IL-6 and IL-1β secretion that in turn polarizes T helper cells towards a proinflammatory Th17 or Th1 phenotype.

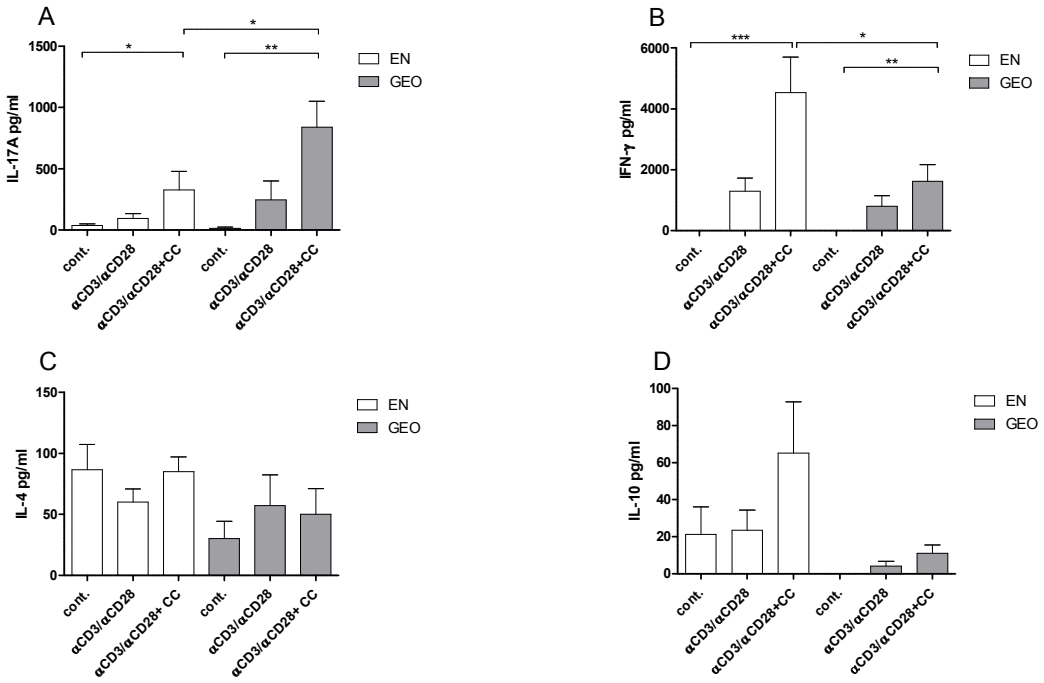

Figure 3.26: Increased IL-17A and IFN-γ secretion in GEO and EN.

Cells from EN (n=5) and GEO (n=5) were left unstimulated (cont.) or stimulated with αCD3/αCD28 in the presence or absence of the chemokine cocktail containing IL-6 and IL-1β (CC: IL-6, 10ng/ml and IL-1β, 5ng/ml). After 7days incubation, supernatants were harvested and the induction of IL-17A(A), IFN-γ (B), IL-4(C) and IL-10(D) were measured by Flowcytomix multiplex assay. Bars indicate mean ± SEM levels of each cytokine. Asterisks show statistical differences (1 way ANOVA and Mann Whitney test) between the groups (*p<0.05, **p<0.01 and ***p<0.001).

4. Discussion

In this study, infection free volunteers living in the endemic area for at least 10 years and were MF negative, had no palpable onchocercomas and no pathology related to onchocerciasis (EN) and infected individuals with either i) palpable nodules, higher worm burden and mild or no pathology (GEO) or ii) less or no palpable nodules, low worm burden and severe pathology (HO) were recruited. The implication of Th1, Th2 and Treg responses in human onchocerciasis is widely investigated but the role of the recently characterized pro-inflammatory Th17 cells in this disease remains unclear. Therefore, the present study aimed at characterizing Th17 cells and investigating whether the balance of Th17/Treg is a possible cause for the different clinical manifestations in human onchocerciasis. Using flow cytometry and PCR array, the data presented in chapter 3 showed that hyperreactive individuals have elevated Th17 responses associated with Th2 responses, whereas GEO individuals exhibited a strong regulatory phenotype and endemic normals presented elevated Th1 responses. To support these findings, filarial-specific responses in these groups were also investigated *in vitro* using an antigen extract prepared from *Onchocerca volvulus* worms. Furthermore, the mechanisms underlining differences between GEO and HO individuals was studied. These data have provided evidence that the adult worms may down-regulate Th17 responses in GEO individuals which is not possible in the HO group since these individuals have very low or even no adult worms. In the final section, the transformation of a GEO phenotype to a HO phenotype was investigated after stimulating cells from GEO and EN individuals with TLRs and NLRs ligands. Overall, the study demonstrated that the regulatory phenotype of GEO patients

could be switched to a pro-inflammatory Th17 state upon additional triggering of innate receptors. In this section, all findings and observations are discussed.

4.1. Immune cell profiles in hyperreactive *Onchocerca volvulus*-infected individuals

Hyperreactive onchocerciasis cases are extremely infrequent, approximately 1% of the infected population and the underlying etiology remains unclear [12, 139]. In this study, the cellular immune profiles of GEO, HO and EN individuals was compared and provided initial evidence that an accentuated Th2/Th17 phenotype forms part of the immune network which the drives hyperreactive state. Th1 cells on the other hand are clearly no longer involved at this stage of pathogenesis since T-bet$^+$ and IFN-γ producing CD4$^+$ T cells were observed at significantly lower levels when compared to EN. The association of IFN-γ and putative immunity in endemic-residing individuals has been demonstrated in studies investigating reactions to L3 larvae [70]. On the other hand, Cooper et al., demonstrated that early exposure to *O. volvulus* infection elicited elevated IFN-γ responses to OvAg but not L3 larvae [53]. Since recent reports have suggested that *Wolbachia,* the endosymbiotic bacteria in *O. volvulus* and other filarial species, were the principal activator of innate and Th1 inflammatory immunity [35], these responses may stem from exposure to worms and/or bacteria.

Previous studies have noted that HO individuals present elevated numbers of peripheral leucocytes and eosinophils but not neutrophils [94, 140]. Moreover, an inverse chemotactic responsiveness was determined in eosinophils and neutrophils in HO patients supporting the hyporesponsive phenotype in GEO patients [140]. Upon analysis of other immune cell subsets *Brattig et al*. [94], found no differences in CD19$^+$ B cells and expanding on those findings we now observed

no alterations in the frequencies of memory B cells ($CD19^+CD27^+$) either. GEO individuals did present however reduced numbers of $CD8^+$ T cells, NK and NKT cells when compared to EN. Due to a higher worm burden in GEO individuals and thus increased amounts of circulating helminth-derived glycolipids and glycoproteins, there may be increased migration of NKT cells into the skin resulting in decreased numbers in blood. HO and GEO individuals on the other hand displayed lower numbers of $CD16^{bright}CD56^{dim}$ and $CD16^{dim}CD56^{bright}$ NK cells. Different combinations of CD56 and CD16 on NK cells can reflect their function. For example, studies have shown that whereas $CD56^{dim}$ cells have high cytotoxic activity, $CD56^{bright}$ cells produce cytokines such as IFN-γ. Thus, *O. volvulus*-infected individuals had reduced populations of both NK fractions. In contrast, EN individuals presented elevated IFN-γ producing T cells and Ov-specific re-stimulation and therefore, further studies may reveal that the elevated $CD16^{dim}CD56^{bright}$ NK cells are producing IFN-γ too. The actual role of NK cells in extracellular parasitic infections, including filarial infections, is not well-defined although studies with the murine model of filariasis, *Litomosoides sigmodontis*, have shown that depletion of NK cells enhanced worm load and Th2 responses at the site of infection [141]. Previous *in vitro* investigations using PBMCs from healthy individuals demonstrated that NK activation and consequential apoptosis resulted from contact with IL-12 producing monocytes after stimulation with filarial antigens [142]. Therefore, the marked increase of monocytes in HO individuals observed in this study could be initiated by i) an elevated requirement of phagocytosis due to increased apoptotic material, ii) increased stimulation due to dying or dead filarial material or iii) simply elevated APC requirement due to elevated hyperreactivity.

An elevated requirement of antigen presentation in HO individuals would correlate to their marked increase in memory $CD4^+$ T cells ($CD4^+CD45RO^+$), an immunological difference not previously reported between the two polar versions of *O. volvulus* infection. In association, $CD45RO^+$ cells were observed via immuno-histochemistry in nodules from sowda individuals [139]. Correspondingly, there was a significant decrease in $CD4^+CD45RA^+$ T cells in HO patients when compared to GEO and EN individuals. Previously, Freedman et al., reported a similar finding in MF^+ *O. volvulus*-infected individuals, which significantly decreased after ivermectin therapy [118]. Further studies also showed increased IFN-γ and IL-5 but decreased IL-10 production in filarial-stimulated PBMC cultures from GEO individuals following therapy [102]. IL-10 plays a substantial role in mediating immune responses, suppressing the growth and function of numerous cells types [143]. As mentioned previously, elevated IL-10 levels are synonymous with a regulatory phenotype (high IgG4 and increased Treg) in GEO individuals [6, 144]. Comparisons of Ov-stimulated PBMCs from MF^+ and amicrofilaridermic (aMF) patients revealed that cell cultures from MF^+ patients made more IL-10 than those isolated from a-MF individuals. Furthermore, following regression analysis this was shown to be associated with the presence of MF and the number of times an individual had received ivermectin [51]. The study presented here is the first to compare the cytokine production of PBMCs from GEO and HO individuals following re-stimulation *in vitro*. Interestingly, although IL-5 and IL-13 levels were markedly increased in cultures from HO individuals, levels of IL-10 were equal. However, via flow cytometry, it was revealed that GEO individuals had a much higher frequency of $IL\text{-}10^+CD4^+$ T cells than HO. $CD4^+$ T cells have been shown to be the largest producers of IL-10 in *O. volvulus*-infected individuals and although

nearly a fifth of those cells further secreted IL-4 hardly any produced IFN-γ. Moreover, IL-10-producing Tr1 cells cloned from subcutaneous tissue surrounding the onchocercomas secreted TGF-β but failed to produceIL-2 and only low levels of IL-4 and IFN-γ if at all [144]. Moreover, the observed hyporesponsiveness was directed towards OvAg and not antigens derived from other filarial species indicating T cell mediated Ov-specific down regulation. Interestingly, the addition of IL-10 or TGF-β neutralizing antibodies to OvAg stimulated PBMCs cultures significantly elevated the proliferative responses in GEO individuals [53, 144].

In association with higher IL-10 and strong regulatory phenotype, $Foxp3^+$ and TGF-β^+ cells have been identified *in situ* within nodules of GEO but not HO individuals via immuno-histochemistry [69, 145], Upon analysis of Treg, the number of $Foxp3^+$ T cells in the $CD4^+$ T cell compartment was higher in HO individuals but analysis of $CD25^{hi}Foxp3^+$ T cell subsets revealed a higher number of these Treg in GEO patients [146, 147]. These data imply therefore, that HO individuals have elevated $CD4^+Foxp3^+$ conventional T cells that are perhaps not regulatory [148]. Interestingly, in correlation, gene expression levels of *Foxp3* in the PCR array were also higher in the HO individuals and here, cells were not stimulated with the stimulation cocktail. These data reflect the hyperresponsive profiles of these patients. To date it remains unclear whether naturally-occurring or induced Treg are required for steering or regulating human filarial infection. Alongside, higher levels of IL-10, GEO individuals are also associated with IgG4 secretion [129]. The propensity to drive IgG4 production has been shown to be mediated by $Foxp3^+$Treg [120] and moreover, was shown to be dependent on GITR-GITRL interaction, IL-10 and TGF-β [120]. In further studies it was shown that prior activation of B cells with TLR4 and TLR9 but not TLR2 hindered the

ability of Treg to dampen Ig secretion in B cells and promoted the production of IL-6 and IL-1β. Moreover, under inflammatory conditions, Treg retained Foxp3 expression but up-regulated RORC2 and secreted IL-17A [130]. Therefore, during microbial infection, B cells could potentially escape Treg control which provides an explanation as to why patients suffering from allergy or helminth disease display polar immunological symptoms despite being exposed to the same agent.

4.2. Elevated balance of Th17/Treg in HO individuals

A central focus of this thesis work was to investigate whether there were changes in the balance between T helper responses and regulatory T cells in the polar forms of onchocerciasis. As anticipated, a prominent Treg phenotype was associated with GEO individuals whereas Th1 and Th2 responses were more dominant in EN. Interestingly, the ratio of Th17/Treg, Th17/Th2 and Th17/Th1 populations were increased in HO individuals compared EN and GEO. The implication of Th17 in clinical manifestations in onchocerciasis has not been clearly delineated. In this study, Th17 cells were shown to be dominant in HO individuals. This suggests that the balance Th17/Treg could play an important role in the pathological outcome of human onchocerciasis.

4.3. Confirmation of the dominant Th17/Th2 phenotype through gene analysis.

The predominance of Th17 responses in patients with severe pathology was confirmed by the significantly higher expression of RORC2 and IL-17 in $CD4^+$ T cells from these patients at both protein and genomic levels. Th17 cell lineages are promoted by the inhibition of Foxp3 by IL-6 and simultaneous elevation of TGF-β and IL-1β responses. IL-1β, especially in synergy with IL-23, plays an essential role in the induction and expansion of murine and human Th17 cells

[135, 149]. Using a Th17 based PCR array, all of these essential Th17-related genes were up-regulated in HO individuals. These included IL-17 family members and IL-22, IL-23A, IL-21. More interestingly, the Th17 transcription factor and signalling pathway molecule RORC2 and STAT3 genes were also up-regulated. In contrast, CCR6 which is known to be important for Th17 cell homing [150] was down-regulated in HO individuals. Further investigation will elucidate these findings. Interestingly, a suppressor of cytokine signaling (SOCS3) gene that has been shown to suppress STAT3 pathway and therefore inhibit Th17 cell commitment [151] was down-regulated in HO compared to GEO. Tregs associated genes such us CCL1 and IL-7R were also under-expressed in HO. Indeed, IL-7R signaling contributes to Treg cell development and peripheral homeostasis [152] and chemokine ligand 1 (CCL1) is critical for the maintenance of Treg function [153]. In correlation with elevated Th1 responses in GEO compared to HO, Th1-related genes such as IL-12β and IL-12Rβ were overregulated in GEO compared to HO. These findings suggest that the implication of Th17 cells in the pathology of human onchocerciasis is regulated at genomic level.

4.4. *Onchocerca volvulus* antigen-specific responses in infected individuals

A major finding in this present study is the dominant Th17/Th2 phenotype in HO individuals. Indeed, upon re-stimulation with Ov antigen, PBMCs from HO patients secreted significant amounts of IL-5 and especially IL-13. In contrast, IL-4 was not detectable in the cell culture supernatants and its expression in $CD4^+$ T cells after intracellular staining did not reflect the phenotype observed in HO individuals after direct FACS analysis. This could be due to: i) IL-4, as a growth factor, was immediately used by the cells, ii) the strong activation with PMA boosts the

transcription factor but not the cytokine secretion or iii) since cells from HO individuals are hyperreactive, a negative feedback control at the transcriptional level could occur after *in vitro* activation of the cells. These data correlate to earlier studies showing an increased likelihood of developing sowda in persons carrying the Arg110 variant of IL-13 which leads to higher IL-13 signalling [154]. Th2 responses in filarial infections have been linked to infection resistance [154, 155] and MF elicit pro-inflammatory responses when they are degenerated or moribund [156]. Thus, a potential scenario for developing hyper-responsiveness may be that deviated Th2 responses provoke MF death which in turn induces a Th17 phenotype (Figure 4.1). In fact, IL-17A-secreting $CD4^+$ T cells were 4 times higher in HO individuals when compared to either EN or GEO groups. Interestingly, although IL-17A, IL-22 and IL-1β responses were low upon Ov antigen stimulation, levels were significantly higher in cell cultures from HO individuals upon TCR activation. The increase of IL-6, IL-22 and IL-1β upon αCD3/αCD28 stimulation could favour the commitment of Th17 responses since IL-17A was also strongly induced after TCR activation. The induction of IL-6 after activation with OvAg may indicate that the parasite could indirectly induce Th17 cell development via the IL-6 pathway. These data indicate that all Th responses (Th1/Th2/Th17) are uncontrolled in HO patients when compared to GEO groups.

In lymphatic filariasis, Th17 cells have been implicated in lymphatic inflammation and are prominent in patients who have cleared bloodstream MF [157]. Indeed, increased antigen-specific Th17 responses were observed in individuals with chronic lymphoedema [116]. Since patients with elevated Th17 responses in LF have less circulating MF it raises the question as to whether MF directly down-regulate Th17 responses to extend their survival. A recent study on *Schistosoma*

haematobium-infected individuals has also revealed an association between Th17 responses and enhanced bladder pathology [115]. Nevertheless, despite the association of pathology and Th17 cells, our findings differ from those studies in two regards. First, patients presenting filarial lymphoedema, had elevated Th1 and Th17 responses following filarial-specific re-stimulation but no alteration in the amount of secreted IL-10 [116]. In the study of *S. haematobium*-infected children [115], Tregs frequencies were equal amongst the infected and control groups whereas in the HO cohort studied here, individuals had reduced numbers of IL-$10^{+}CD4^{+}$ and $CD25^{hi}Foxp3^{+}CD4^{+}$ Tregs when compared to the GEO group. In association, asymptomatic *W. bancrofti*-infected individuals also presented higher levels of $Foxp3^{+}$ on the mRNA level but only after re-stimulation with filarial antigen [116]. Thus, although Th17-producing cells appear to be a common dominator in helminth-infected individuals displaying severe pathology each infection appears to have created its own subtle collaboration of immune parameters such as Treg, IL-10 and in HO accentuated Th2 responses as well. OvAg and αCD3/αCD28 activation did not induced IL-10 secretion in $CD4^{+}$ T cells. This is not in line with the literature since Infection with filarial parasites is associated with T cell hyporesponsiveness, which is thought to be partly mediated by their ability to induce regulatory T cells (Treg) during human infections and also filarial-specific IL-10 was pointed to play a role in the suppression of effector T cells response [111, 112, 146, 158]. T cell receptor activation showed increase IL-5, IL-13 and IFN-γ production in cultures from the HO group when compared to GEO individuals, this could be explained by: i) hyperresponsiveness state and ii) modified Th1 or Th2 responses. In association with the data shown in Figure 3.2A, cells from EN secreted significantly more IFN-γ upon activation with Ov-antigen

and levels were significantly higher than those produced by cells from HO individuals. However, the dampened IFN-γ responses from cells of HO individuals was not reflected upon αCD3/αCD28 activation since here significantly higher levels were observed when compared to both EN and GEO groups, indicating that failure to produce IFN-γ was not a deficit of Th1 cells per se but dampened filarial-specific IFN-γ-producing cells (Figure 3.7D).

In order to confirm the antigen-specific Th cells cytokine profiles observed in cell culture supernatants and since IL-4 was not detectable, OvAg-activated PBMCs from different study groups were stained intracellulary for IFN-γ, IL-4, IL-17A and IL-10. All these cytokines could be observed via flow cytometry analysis but did not correlate with cell culture supernatant data.

Another novelty and interesting finding in this study was the increased antigen-specific IL-9 secretion in HO individuals upon stimulation with OvAg. Indeed, interleukin-9 is believed to induce Th17 cell differentiation, enhance the function of natural regulatory T cells; hence plays an important role in pro-inflammatory diseases and the regulation of the immune system [159, 160]. The induction of Th9 cells in EN and HO may indicate that these cells are involved in the clearance of the parasite since GEO individuals that harbor higher worm burdens fail to induce OvAg-specific Th9 responses. This is in concordance with the literature since the combination of Th17 and Th9 responses were shown in inflammatory conditions [134, 161] and also Th17 cells were shown to secrete IL-9 [162, 163].

4.5. Antibodys profiles in *Onchocerca volvulus*-infected individuals

Antibody-mediated immunity plays an important role in helminth infection and is also associated with different clinical manifestation [164]. Therefore, antibodiy

profiles were assessed in the cell culture supernatants of HO individuals against EN and GEO. The role of IgA and IgM in hyperreactive onchocerciasis is not well investigated. Here, IgA and IgM were shown to be linked in the elevated pathology of human onchocerciasis. Further investigations are needed however to completely define the function or activity of these antibodies in filariasis. The combination of Th2 and Th17 responses might activate B cells to preferentially produce IgA and IgM. This also has to be further investigated. In this study, HO individuals presented increased levels of the Th17-related cytokine IL-6 which has been shown to induce IgA and IgM secretion by B cells [165, 166]. Interestingly, IL-17A was shown to be determinant in antibody production by B cells but the exact role remains unclear [167].

Upon OvAg stimulation, IgG2, IgG4, IgA and IgM were increased in cultures from HO individuals when compared to levels in cultures from EN and GEO groups. Interestingly, increased spontaneous release of IgG4 and IgE was observed in cultures from GEO and HO individuals respectively while IgG3 was high in just the "medium" or control supernatant of EN. Taken together, these results suggest that IgG2 and IgE are not only associated with the clearance of the parasite in HO individuals but could lead to the skin inflammation observed in this group. Base line release of IgG4 in GEO could explain the regulatory state of these individuals, while IgG3 in the "medium" of EN-derived cell cultures may be associated with protection and immunity against the larval stage. Indeed, It has been reported that in onchocerciasis, FC receptor and complement-binding IgG3 levels are higher in EN compared with those in GEO who are characterized by elevated IgG4 antibodies [168]. In contrast, high sera levels of IgE, IgG1, IgG2, IgG3 were observed in sowda individuals (HO) [169].

A new finding associated with this study is the increased levels of OvAg-specific IgM and IgA that seems to be associated with the clearance of the parasites, an area which warrants further investigation in Onchocerca infections. Elevated concentrations of IgM and IgA were observed in sera from Onchocerca volvulus-infected patients from Sudan [170].

4.6. OvAg modulated Th17 cells polarization *in vitro*

The immunomodulatory properties of helminth-derived antigens is well documented [171]. Here we found that individuals with high MF load and adult worms (GEO) have diminished Th17 responses while people with less or no MF and adult worms (HO) develop strong Th17 responses. The question was whether adult worms, in order to survive, down-regulate Th17 responses in GEO people. To test this, adult worm antigen extract was use *in vitro* to block Th17 cell polarization. The development and function of Th17 cells requires TCR activation in the presence of cytokines such as IL-6 and IL-1β [133]. In this study, it was found that the combination of IL-6 and IL-1β (CC) upon TCR activation increased IL-17A secretion. This higher induction of IL-17A was down-regulated when OvAg was added to the system. In addition, Th2 cytokines were also down-regulated but there was an increase in IL-10 and IFN-γ secretion. This suggests the potential of adult worm antigen to modulate not only the development of Th17 responses but also the Th2 responses. Indeed, *O. volvulus* antigen components have been implicated in the down-regulation of effector actions of both Th1 and Th2 responses [172]. The induction of regulatory cells that produce IL-10 and/or TGF-β has been demonstrated in several studies to be the mechanism used by helminths to regulate the immune system [173]. Here, we observed that OvAg increased Th1 cytokines and IL-10 in the presence of CC. Therefore, we hypothesized that the

modulation of Th17 cells polarization was through Th1, IL-10 and Th2 pathway although IL-13 was down-regulated in the presence of OvAg. The addition of rhIL-4, rhIL-13, rhIL-10 or all together in the absence of OvAg decreased IL-17A secretion. This is in line with literature since Th2 and Th17 responses are known to be antagonist [174]. *Rui He et al.* demonstrated that in the absence of Th2 cytokines, IL-17A responses to epicutaneous sensitization mediated airway inflammation was exaggerated [175]. In contrast, the addition of anti-IL-4, anti-IL-13 and anti-IL-10 in the presence of OvAg did not restore the secretion of IL-17A suggesting that OvAg used neither IL-4, IL-13 nor IL-10 pathways to modulate Th17 cell development. To further elucidate this phenomenon, it was hypothesized that OvAg may directly act on T cells, or indirectly on antigen-presentation cells (APC). Therefore, T cells, alone or T cells in co-culture with B cells or monocytes were activated with αCD3/αCD28 in the presence of CC and OvAg. It was observed that when cultured alone or in presence of B cells, OvAg did not down-regulate the induction of IL-17A in CC. However, OvAg could down-regulate IL-17A secretion in T-monocytes co-culture. This indicates that OvAg may suppress the antigen presenting capacity of monocytes to modulate Th17 cell development.

4.7. Additional innate receptors stimulation switch GEO phenotype to HO

In *O. volvulus* endemic areas, the existence of the two polar forms of the disease is still not fully understood. The question was therefore, whether there is a group of people who can only develop GEO or HO phenotype or is there a dynamic switch from GEO to HO? Soboslay *et al*. and others have demonstrated that children born from infected mothers develop GEO while those from non-infected mother manifest severe pathology form of the disease, suggesting a maternal imprinting of the permissiveness [8, 176]. Gene polymorphism influences clinical manifestation

in helminth infection and immune heterogeneity arises not from hosts and parasites in isolation, but also from the environment in which immune responses develop [177]. For instance VEGF-A gene polymorphism was shown to be associated with the development of hydrocele in lymphatic filariasis [178]. In human onchocerciasis, Fc gamma RIIa gene polymorphism has been implicated in the development of severe reactive onchodermatitis [179]. To study the transformation of GEO to HO, cells from GEO individuals were activated with TLR and NLR ligands. Here it was seen that the stimulation of TLR4, NOD1 and NOD2 could switch a GEO phenotype into a HO one as reflected by the increased expression of IL-17A and IFN-γ cytokines in the GEO group. More interestingly, the addition of recombinant human IL-6 and IL-1β induced a significant increase of IL-17A secretion in cultures from GEO individuals when compared to EN indicating that: i) TLR and NLR activation induced these cytokines which in turn could change a GEO phenotype into a HO one by transforming the existing Tregs in GEO to Th17 cells and ii) *O. volvulus* infection did not suppress the host innate immune system. *In vitro* studies using recombinant IL-12 and IL-18 showed that while IL-18 could reduce antigen-specific IL-10 secretion in cells from *O. volvulus*-infected individuals, IL-12 re-directed antigen responsiveness by promoting IFN-γ production [180]. All together, these observations may suggest that in addition to genetic polymorphism the different polar forms of infection outcomes observed following *O. volvulus* infection are driven by external factors such as bacterial infection that apparently activate innate receptors. Such findings point to the importance of co-infection towards the elimination of filarial infection globally.

4.8. Conclusion

This thesis aimed at characterizing the role of Th17 cells and the assessing whether the balance of Th17/Treg were possible causes in the differential clinical manifestations in human onchocerciasis. The data showed that while GEO individuals are characterized by increased regulatory responses shown by higher $CD4^+CD25^{hi}Foxp3^+$ T cells and IL-10 $CD4^+$ T producing cells, HO individuals exhibited strong Th17 and Th2 responses with elevated ratios of Th17/Treg. Interestingly, the Th1 phenotype was the only characteristic response of EN. The antigen-specific responsiveness of cells from the study groups indicated increased Th1 and Th2 responses in EN and GEO respectively. This work highlights the possible underlying mechanisms between GEO and HO. Here OvAg significantly mediated *in vitro* development of Th17 cells by transforming a GEO phenotype into a HO phenotype following activation of innate receptors which lead to increased production of cytokines such as IL-6 and IL-1β. The study also indicates that the strong Th17 responses observed in PBMCs was not specific to O.v. adult worm antigen. This raises the question whether other components of the worm could induce this response. Therefore there is the need for further investigations into other possible inducers of Th17 responses such as the endosymbiont *Wolbachia* and possibly microfilariae antigen. Also the role of IgM and IgA in onchocerciasis has to be further delineated. Due to the lack of a suitable murine model for the complete life-cycle of *O. volvulus* parasite, the study could not confirm the findings using animal models. For these reasons, the findings might not reflect the whole human immune activity in onchocerciasis. Figure 4.1 represents a model that describes different mechanisms that can drive the

development of Th17 responses in HO individuals and could explain in-depth what makes the difference between HO associated pathology and GEO regulation.

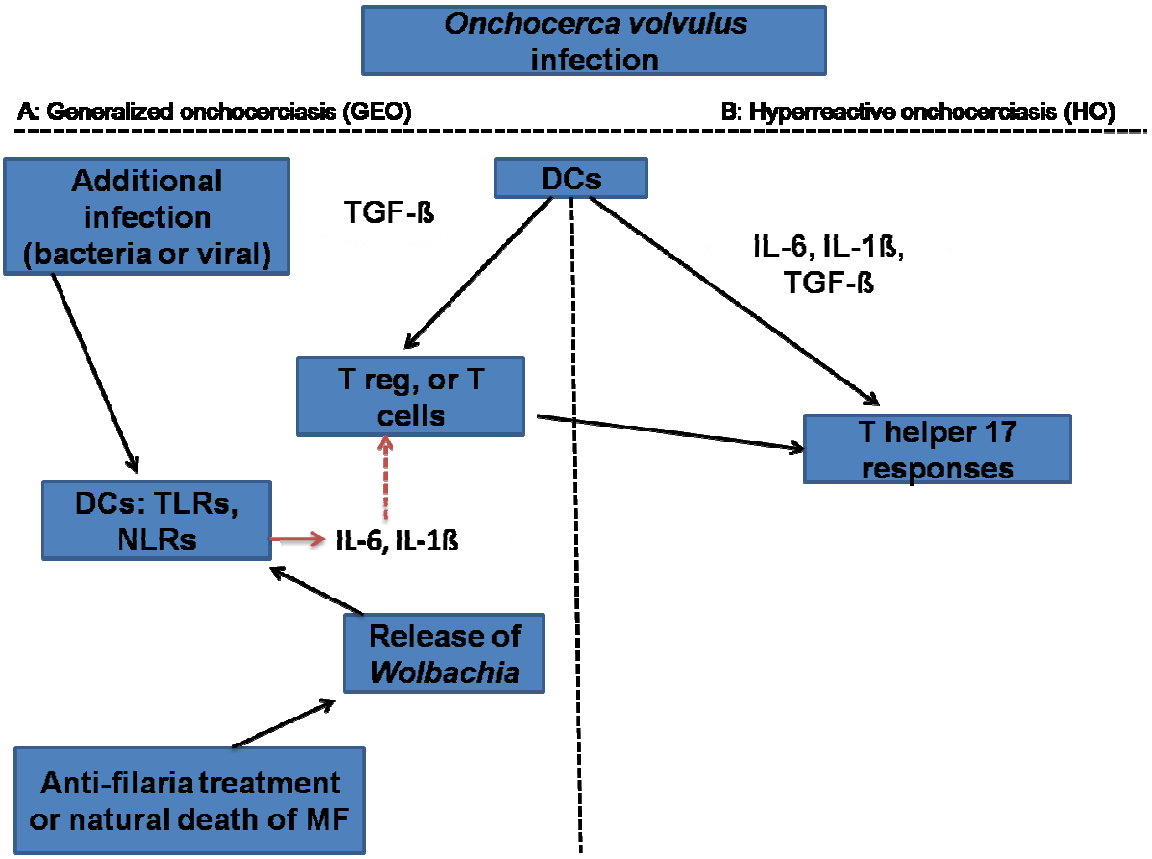

Figure 4.1: Development of T helper 17 responses in *Onchocerca volvulus* infection.

Onchocerca volvulus infection can lead to two polar forms: generalized onchocerciasis (GEO) or hyperreactive onchocerciasis (HO). (A) GEO. Parasites could activate dendritic cells (DCs) to produce TGF-ß that polarize T cells to regulatory T cells (Treg). Additional infection or the release of bacterial endosymbiont *Wolbachia* after anti-filaria treatment could activate Toll-like receptors (TLRs) or Nod-like receptors (NLRs) on DCs to release IL-6 and IL-1ß that may transform Treg or naive helper T cells to Th17. (B) HO. In addition to the latter pathway, the parasite can stimule DCs to release IL-6, IL-1ß and TGF-ß that could polarize T helper cells toward Th17 responses.

5. References

1. Bandi, C., et al., *Phylogeny of Wolbachia in filarial nematodes.* Proceedings of the Royal Society of London. Series B: Bio Sci, 1998. **265**(1413): p. 2407-2413.
2. Blaxter, M.L., et al., *A molecular evolutionary framework for the phylum Nematoda.* Nat, 1998. **392**(6671): p. 71-75.
3. Dieterich, C. and R.J. Sommer, *How to become a parasite – lessons from the genomes of nematodes.* Trends Genet, 2009. **25**(5): p. 203-209.
4. Brattig, N.W., *Pathogenesis and host responses in human onchocerciasis: impact of Onchocerca filariae and Wolbachia endobacteria.* Micro Infect, 2004. **6**(1): p. 113-128.
5. Hartmann, S. and R. Lucius, *Modulation of host immune responses by nematode cystatins.* Int J Parasitol, 2003. **33**(11): p. 1291-1302.
6. Adjobimey, T. and A. Hoerauf, *Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation.* Ann Trop Med Parasitol, 2010. **104**(6): p. 455-64.
7. Hoerauf, A. and N. Brattig, *Resistance and susceptibility in human onchocerciasis – beyond Th1 vs Th2.* Trends Parasitol, 2002. **18**(1): p. 25-31.
8. McSorley, H.J. and R.M. Maizels, *Helminth Infections and Host Immune Regulation.* Clin Microbiol Rev, 2012. **25**(4): p. 585-608.
9. Pearlman, E., *Immunopathogenesis of Onchocerca volvulus keratitis (river blindness): a novel role for endosymbiotic Wolbachia bacteria.* Med Microbiol Immunol, 2003. **192**(1): p. 57-60.
10. Taylor, M.J., A. Hoerauf, and M. Bockarie, *Lymphatic filariasis and onchocerciasis.* Lancet, 2010. **376**(9747): p. 1175-85.
11. Bandi, C., A.J. Trees, and N.W. Brattig, *Wolbachia in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases.* Vet Parasitol, 2001. **98**(1–3): p. 215-238.

12. Hotez, P.J., et al., *Helminth infections: the great neglected tropical diseases.* J Clin Invest, 2008. **118**(4): p. 1311-1321.

13. Hooper, P.J., et al., *The Global Programme to Eliminate Lymphatic Filariasis: health impact during its first 8 years (2000–2007).* Ann Trop Med Parasitol, 2009. **103**(Supplement-1): p. 17-21.

14. Molyneux, D.H., et al., *Mass drug treatment for lymphatic filariasis and onchocerciasis.* Trends Parasitol, 2003. **19**(11): p. 516-522.

15. *Research priorities for helminth infections*: World Health Organ Tech Rep Ser. 2012;(972):xv-xvii, 1-174, back cover.

16. Boatin, B.A. and F.O. Richards Jr, *Control of Onchocerciasis*, in *Advances in Parasitology*, H.M. David, Editor. 2006, Academic Press. p. 349-394.

17. Orihel, T.C. and M.L. Eberhard, *Zoonotic Filariasis.* Clin Microbiol Rev, 1998. **11**(2): p. 366-381.

18. Morales-Hojas, R., R.A. Cheke, and R.J. Post, *Molecular systematics of five Onchocerca species (Nematoda: Filarioidea) including the human parasite, O. volvulus, suggest sympatric speciation.* J Helminthol, 2006. **80**(03): p. 281-290.

19. Schulz-Key, H., *Observations on the reproductive biology of Onchocerca volvulus.* Acta Leidensia, 1990. **59**(1-2): p. 27-44.

20. *Meeting of the International Task Force for Disease Eradication, January 2014.* Wkly Epidemiol Rec, 2014. **89**(15): p. 153-60.

21. Diseases, W.H.O.C., *Certification of Elimination of Human Onchocerciasis: Criteria and Procedures*. 2001: World Health Organization, Communicable Diseases.

22. *Progress toward elimination of onchocerciasis in the Americas - 1993-2012.* MMWR Morb Mortal Wkly Rep, 2013. **62**(20): p. 405-8.

23. Hall, L.R. and E. Pearlman, *Pathogenesis of onchocercal keratitis (River blindness).* Clin Microbiol Rev, 1999. **12**(3): p. 445-53.

24. Udall, D.N., *Recent updates on onchocerciasis: diagnosis and treatment.* Clin Infect Dis, 2007. **44**(1): p. 53-60.

25. Albers, A., et al., *Retarded Onchocerca volvulus L1 to L3 larval development in the Simulium damnosum vector after anti-wolbachial treatment of the human host.* Parasit Vectors, 2012. **5**: p. 12.

26. Hoerauf, A.M., ed. *Onchocerciasis, in Tropical Infectious Diseases: Principles, Pathogens and Practice*. ed. D.H.W. R. L. Guerrant, Peter F. Weller. 2010.

27. Maizels, R.M. and R.A. Lawrence, *Immunological tolerance: The key feature in human filariasis?* Parasitol Today, 1991. **7**(10): p. 271-276.

28. van Riet, E., F.C. Hartgers, and M. Yazdanbakhsh, *Chronic helminth infections induce immunomodulation: Consequences and mechanisms.* Immunobiol, 2007. **212**(6): p. 475-490.

29. Taylor, M.J., *Wolbachia in the inflammatory pathogenesis of human filariasis.* Ann N Y Acad Sci, 2003. **990**: p. 444-9.

30. King, C.L. and T.B. Nutman, *Regulation of the immune response in lymphatic filariasis and onchocerciasis.* Immunol Today, 1991. **12**(3): p. A54-A58.

31. Ali, M.M., et al., *Immune Responses Directed against Microfilariae Correlate with Severity of Clinical Onchodermatitis and Treatment History.* J Infect Dis, 2003. **187**(4): p. 714-717.

32. Mpagi, J.L., et al., *Humoral responses to a secretory Onchocerca volvulus protein: differences in the pattern of antibody isotypes to recombinant Ov20/OvS1 in generalized and hyperreactive onchocerciasis.* Parasite Immunol, 2000. **22**(9): p. 455-460.

33. Freedman, D.O., et al., *Lymphoscintigraphic Analysis Of Lymphatic Abnormalities In Symptomatic And Asymptomatic Human Filariasis.* J Infect Dis, 1994. **170**(4): p. 927-933.

34. Tamarozzi, F., et al., *Onchocerciasis: the Role of Wolbachia Bacterial Endosymbionts in Parasite Biology, Disease Pathogenesis, and Treatment.* Clin Microbiol Rev, 2011. **24**(3): p. 459-468.

35. Tamarozzi, F., et al., *Onchocerciasis: the role of Wolbachia bacterial endosymbionts in parasite biology, disease pathogenesis, and treatment.* Clin Microbiol Rev, 2011. **24**(3): p. 459-68.

36. Mackenzie, C.D., et al., *Variations in Host Responses and the Pathogenesis of Human Onchocerciasis.* Rev Infect Dis, 1985. **7**(6): p. 802-808.

37. Enk, C.D., *Onchocerciasis—river blindness.* Clinics in Dermatology, 2006. **24**(3): p. 176-180.

38. Fawdry, A.L., *Onchocerciasis in South Arabia.* Transactions of The Royal Society of Tropical Med Hyg, 1957. **51**(3): p. 253-256.

39. Bartlett, A., et al., *Variation in delayed hypersensitivity in onchocerciasis.* Trans R Soc Trop Med Hyg, 1978. **72**(4): p. 372-7.

40. Ghalib, H.W., et al., *Severe onchocercal dermatitis in the Ethiopian border region of Sudan.* Ann Trop Med Parasitol, 1987. **81**(4): p. 405-19.

41. Murdoch, M.E., et al., *A clinical classification and grading system of the cutaneous changes in onchocerciasis.* Br J Dermatol, 1993. **129**(3): p. 260-269.

42. Brattig, N.W., et al., *Immunohistology of ectopic secondary lymph follicles in subcutaneous nodules from patients with hyperreactive onchocerciasis (sowda).* Parasitol Res, 2010. **107**(3): p. 657-66.

43. Mand, S., et al., *Frequent detection of worm movements in onchocercal nodules by ultrasonography.* Filaria J, 2005. **4**(1): p. 1.

44. Hoerauf, A., et al., *Wolbachia endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study.* Med Microbiol Immunol, 2008. **197**(3): p. 295-311.

45. Chan, C.-C., et al., *Immunopathology of Ocular Onchocerciasis: 2. Anti-retinal Autoantibodies in Serum and Ocular Fluids.* Ophthalmol, 1987. **94**(4): p. 439-443.

46. Plier, D.A., K. Awadzi, and D.O. Freedman, *Immunoregulation in Onchocerciasis: Persons with Ocular Inflammatory Disease Produce a Th2-like Response to Onchocerca volvulus Antigen.* J Infect Dis, 1996. **174**(2): p. 380-386.

47. Remme, J., et al., *Ocular onchocerciasis and intensity of infection in the community. I. West African savanna.* Trop Med Parasitol, 1989. **40**(3): p. 340-7.

48. Dadzie, K.Y., et al., *Ocular onchocerciasis and intensity of infection in the community. III. West African rainforest foci of the vector Simulium sanctipauli.* Trop Med Parasitol, 1990. **41**(4): p. 376-82.

49. Bradley, J.E., et al., *River blindness: a role for parasite retinoid-binding proteins in the generation of pathology?* Trends Parasitol, 2001. **17**(10): p. 471-475.

50. Katabarwa, M.N., et al., *Does onchocerciasis transmission take place in hypoendemic areas? a study from the North Region of Cameroon.* Trop Med Int Health, 2010. **15**(5): p. 645-652.

51. Arndts, K., et al., *Immunoepidemiological profiling of onchocerciasis patients reveals associations with microfilaria loads and ivermectin intake on both individual and community levels.* PLoS Negl Trop Dis, 2014. **8**(2): p. e2679.

52. Hoerauf, A., et al., *Depletion of Wolbachia endobacteria in Onchocerca volvulus by docycycline and microfilaridermia after ivermectin treatment.* Lancet, 2001. **357**: p. 1415 - 1416.

53. Cooper, P.J., et al., *Early Human Infection with Onchocerca volvulus Is Associated with an Enhanced Parasite-Specific Cellular Immune Response.* J Infect Dis, 2001. **183**(11): p. 1662-1668.

54. Weil, G.J., et al., *A Rapid-Format Antibody Card Test for Diagnosis of Onchocerciasis.* J Infect Dis, 2000. **182**(6): p. 1796-1799.

55. Lobos, E., et al., *An immunogenic Onchocerca volvulus antigen: a specific and early marker of infection.* Sci, 1991. **251**(5001): p. 1603-1605.

56. Ramachandran, C.P., *Improved immunodiagnostic tests to monitor onchocerciasis control programmes — A multicenter effort.* Parasitol Today, 1993. **9**(3): p. 77-79.

57. Hoerauf, A., et al., *Filariasis in Africa—treatment challenges and prospects.* Clinical Microbiol Infect, 2011. **17**(7): p. 977-985.

58. Vieira, J., et al., *Impact of long-term treatment of onchocerciasis with ivermectin in Ecuador: potential for elimination of infection.* BMC Med, 2007. **5**(1): p. 9.

59. Boussinesq, M., J. Prod'hon, and J.P. Chippaux, *Onchocerca volvulus: striking decrease in transmission in the Vina valley (Cameroon) after eight annual large scale ivermectin treatments.* Trans R Soc Trop Med Hyg, 1997. **91**(1): p. 82-86.

60. Hoerauf, A., et al., *Doxycycline in the treatment of human onchocerciasis: Kinetics of Wolbachia endobacteria reduction and of inhibition of embryogenesis in female Onchocerca worms.* Microbes Infect, 2003. **5**(4): p. 261-73.

61. Wanji, S., et al., *Community-directed delivery of doxycycline for the treatment of onchocerciasis in areas of co-endemicity with loiasis in Cameroon.* Parasit Vectors, 2009. **2**(1): p. 1756-3305.

62. Hoerauf, A., et al., *Efficacy of 5-week doxycycline treatment on adult Onchocerca volvulus.* Parasitol Res, 2009. **104**(2): p. 437-47.

63. Taylor, M.J., et al., *Anti-Wolbachia drug discovery and development: safe macrofilaricides for onchocerciasis and lymphatic filariasis.* Parasitol, 2014. **141**(1): p. 119-27.

64. Hoerauf, A., *Targeting of wolbachia endobacteria in litomosoides sigmodontis: comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxacin.* Trop Med Int Health, 2000. **5**(4): p. 275-9.

65. Hoerauf, A., O. Adjei, and D.W. Buttner, *Antibiotics for the treatment of onchocerciasis and other filarial infections.* Curr Opin Investig Drugs, 2002. **3**(4): p. 533-7.

66. Debrah, A.Y., et al., *Macrofilaricidal effect of 4 weeks of treatment with doxycycline on Wuchereria bancrofti.* Trop Med Int Health, 2007. **12**(12): p. 1433-41.

67. Debrah, A.Y., et al., *Macrofilaricidal Activity in Wuchereria bancrofti after 2 Weeks Treatment with a Combination of Rifampicin plus Doxycycline.* J Parasitol Res, 2011. **2011**: p. 201617.

68. Hoerauf, A., et al., *Doxycycline as a novel strategy against bancroftian filariasis-depletion of Wolbachia endosymbionts from Wuchereria bancrofti and stop of microfilaria production.* Med Microbiol Immunol, 2003. **192**(4): p. 211-6.

69. Korten, S., et al., *Natural death of adult Onchocerca volvulus and filaricidal effects of doxycycline induce local FOXP3+/CD4+ regulatory T cells and granzyme expression.* Microbes Infect, 2008. **10**(3): p. 313-24.

70. Turner, J.D., et al., *Wolbachia Lipoprotein Stimulates Innate and Adaptive Immunity through Toll-like Receptors 2 and 6 to Induce Disease Manifestations of Filariasis.* J Bio Chem, 2009. **284**(33): p. 22364-22378.

71. Allen, J.E. and R.M. Maizels, *Diversity and dialogue in immunity to helminths.* Nat Rev Immunol, 2011. **11**(6): p. 375-388.

72. André, A.v.S., et al., *The Role of Endosymbiotic Wolbachia Bacteria in the Pathogenesis of River Blindness.* Sci, 2002. **295**(5561): p. 1892-1895.

73. Hoerauf, A., et al., *Immunomodulation by filarial nematodes.* Parasite Immunol, 2005. **27**(10-11): p. 417-29.

74. Chomarat, P., et al., *IL-6 switches the differentiation of monocytes from dendritic cells to macrophages.* Nat Immunol, 2000. **1**(6): p. 510-514.

75. Takashiba, S., et al., *Differentiation of Monocytes to Macrophages Primes Cells for Lipopolysaccharide Stimulation via Accumulation of Cytoplasmic Nuclear Factor κB.* Infect Immun, 1999. **67**(11): p. 5573-5578.

76. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity.* Nat Rev Immunol, 2005. **5**(12): p. 953-964.

77. Goerdt, S., et al., *Alternative versus Classical Activation of Macrophages.* Pathobiol, 1999. **67**(5-6): p. 222-226.

78. Rodríguez-Sosa, M., et al., *Chronic Helminth Infection Induces Alternatively Activated Macrophages Expressing High Levels of CCR5 with Low Interleukin-12 Production and Th2-Biasing Ability.* Infect Immun, 2002. **70**(7): p. 3656-3664.

79. Taylor, M.D., et al., *F4/80+ Alternatively Activated Macrophages Control CD4+ T Cell Hyporesponsiveness at Sites Peripheral to Filarial Infection.* J Immunol, 2006. **176**(11): p. 6918-6927.

80. Kreider, T., et al., *Alternatively activated macrophages in helminth infections.* Curr Opin in Immunol, 2007. **19**(4): p. 448-453.

81. Makepeace, B.L., et al., *Granulocytes in helminth infection -- who is calling the shots?* Curr Med Chem, 2012. **19**(10): p. 1567-86.

82. Cadman, E.T. and R.A. Lawrence, *Granulocytes: effector cells or immunomodulators in the immune response to helminth infection?* Parasite Immunol, 2010. **32**(1): p. 1-19.

83. Shin, M.H., Y.A. Lee, and D.Y. Min, *Eosinophil-mediated tissue inflammatory responses in helminth infection.* Korean J Parasitol, 2009. **47**(31).

84. Klion, A.D. and T.B. Nutman, *The role of eosinophils in host defense against helminth parasites.* J Allergy Clin Immunol, 2004. **113**(1): p. 30-37.

85. Moreau, E. and A. Chauvin, *Immunity against Helminths: Interactions with the Host and the Intercurrent Infections.* J Biomed Biotechnol, 2010. **2010**.

86. Klion, A.D. and T.B. Nutman, *The role of eosinophils in host defense against helminth parasites.* J Allergy Clin Immunol, 2004. **113**(1): p. 30-7.

87. Leiferman, K.M., et al., *Dermal Deposition of Eosinophil-Granule Major Basic Protein in Atopic Dermatitis.* N Engl J Med, 1985. **313**(5): p. 282-285.

88. Pearlman, E., et al., *The role of eosinophils and neutrophils in helminth-induced keratitis.* Investig Ophthalmol Vis Sci, 1998. **39**(7): p. 1176-82.

89. Campos-Martin, Y., et al., *Expression of human CD1d molecules protects target cells from NK cell-mediated cytolysis.* J Immunol, 2004. **172**(12): p. 7297-305.

90. Berrou, J., et al., *Natural Killer Cell Function, an Important Target for Infection and Tumor Protection, Is Impaired in Type 2 Diabetes.* PLoS One, 2013. **8**(4): p. e62418.

91. Beziat, V., et al., *CD56brightCD16+ NK cells: a functional intermediate stage of NK cell differentiation.* J Immunol, 2011. **186**(12): p. 6753-61.

92. Faveeuw, C., T. Mallevaey, and F. Trottein, *Role of natural killer T lymphocytes during helminthic infection.* Parasite, 2008. **15**(3): p. 384-388.

93. Babu, S., C.P. Blauvelt, and T.B. Nutman, *Filarial Parasites Induce NK Cell Activation, Type 1 and Type 2 Cytokine Secretion, and Subsequent Apoptotic Cell Death.* J Immunol, 2007. **179**(4): p. 2445-2456.

94. Brattig, N.W., et al., *Distribution pattern of peripheral lymphocyte subsets in localized and generalized form of onchocerciasis.* Clin Immunol Immunopathol, 1987. **44**(2): p. 149-59.

95. Parker, D.C., *T cell-dependent B cell activation.* Annu Rev Immunol, 1993. **11**: p. 331-60.

96. Haas, A., K. Zimmermann, and A. Oxenius, *Antigen-Dependent and -Independent Mechanisms of T and B Cell Hyperactivation during Chronic HIV-1 Infection.* J Virol, 2011. **85**(23): p. 12102-12113.

97. Itano, A.A., et al., *Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity.* Immun, 2003. **19**(1): p. 47-57.

98. Ekkens, M.J., et al., *The role of OX40 ligand interactions in the development of the Th2 response to the gastrointestinal nematode parasite Heligmosomoides polygyrus.* J Immunol, 2003. **170**(1): p. 384-93.

99. Harris, N. and W.C. Gause, *To B or not to B: B cells and the Th2-type immune response to helminths.* Trends Immunol. **32**(2): p. 80-88.

100. Rubio de Kromer, M.T., C.E. Medina-De la Garza, and N.W. Brattig, *Differences in eosinophil and neutrophil chemotactic responses in sowda and generalized form of onchocerciasis.* Acta Trop, 1995. **60**(1): p. 21-33.

101. Nutman, T.B., R. Hussain, and E.A. Ottesen, *IgE production in vitro by peripheral blood mononuclear cells of patients with parasitic helminth infections.* Clin Exp Immunol, 1984. **58**(1): p. 174-82.

102. Henry, N.L., et al., *Onchocerciasis in a nonendemic population: clinical and immunologic assessment before treatment and at the time of presumed cure.* J Infect Dis, 2001. **183**(3): p. 512-6.

103. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.

104. Luckheeram, R.V., et al., *CD4+T Cells: Differentiation and Functions.* Clin Dev Immunol, 2012. **2012**: p. 12.

105. Ottesen, E.A., *Immune Responsiveness And The Pathogenesis Of Human Onchocerciasis.* J Infect Dis, 1995. **171**(3): p. 659-671.

106. Elson, L.H., et al., *Immunity to onchocerciasis: putative immune persons produce a Th1-like response to Onchocerca volvulus.* J Infect Dis, 1995. **171**(3): p. 652-8.

107. Soboslay, P.T., et al., *The diverse expression of immunity in humans at distinct states of Onchocerca volvulus infection.* Immunol, 1997. **90**(4): p. 592-9.

108. Doetze, A., et al., *Production of both IFN-gamma and IL-5 by Onchocerca volvulus S1 antigen-specific CD4+ T cells from putatively immune individuals.* Int Immunol, 1997. **9**(5): p. 721-9.

109. Turaga, P.S., et al., *Immunity to onchocerciasis: cells from putatively immune individuals produce enhanced levels of interleukin-5, gamma interferon, and granulocyte-macrophage colony-stimulating factor in response to Onchocerca volvulus larval and male worm antigens.* Infect Immun, 2000. **68**(4): p. 1905-11.

110. Timmann, C., et al., *Cutaneous pathology in onchocerciasis associated with pronounced systemic T-helper 2-type responses to Onchocerca volvulus.* Br J Dermatol, 2003. **149**(4): p. 782-787.

111. Satoguina, J., et al., *Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis).* Microbes Infect, 2002. **4**(13): p. 1291-300.

112. Velavan, T. and O. Ojurongbe, *Regulatory T Cells and Parasites.* J Biomed Biotechnol, 2011. **2011**: p. 8.

113. Hoerauf, A., et al., *Immunomodulation by filarial nematodes.* Parasite Immunol, 2005. **27**(10-11): p. 417-429.

114. Mitre, E., D. Chien, and T.B. Nutman, *CD4(+) (and not CD25+) T cells are the predominant interleukin-10-producing cells in the circulation of filaria-infected patients.* J Infect Dis, 2008. **197**(1): p. 94-101.

115. Mbow, M., et al., *T-helper 17 cells are associated with pathology in human schistosomiasis.* J Infect Dis, 2013. **207**(1): p. 186-95.

116. Babu, S., et al., *Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells.* PLoS Negl Trop Dis, 2009. **3**(4): p. e420.

117. Stewart, G.R., et al., *Onchocerciasis modulates the immune response to mycobacterial antigens.* Clin Exp Immunol, 1999. **117**(3): p. 517-23.

118. Freedman, D.O., et al., *Immunoregulation in onchocerciasis. Functional and phenotypic abnormalities of lymphocyte subsets and changes with therapy.* J Clin Invest, 1991. **88**(1): p. 231-8.

119. Ward, J., et al., *Onchocerciasis and Immunity in Humans: Enhanced T Cell Responsiveness to Parasite Antigen in Putatively Immune Individuals.* J Infect Dis, 1988. **157**(3): p. 536-543.

120. Satoguina, J.S., et al., *Tr1 and naturally occurring regulatory T cells induce IgG4 in B cells through GITR/GITR-L interaction, IL-10 and TGF-beta.* Eur J Immunol, 2008. **38**(11): p. 3101-13.

121. Kaneko, M., et al., *Allergen-specific IgG1 and IgG3 through Fc gamma RII induce eosinophil degranulation.* J Clin Invest, 1995. **95**(6): p. 2813-21.

122. Wamae, C.N., et al., *Kinetics of circulating human IgG4 after diethylcarbamazine and ivermectin treatment of bancroftian filariasis.* J Infect Dis, 1992. **165**(6): p. 1158-60.

123. Taylor, M.D., et al., *Removal of Regulatory T Cell Activity Reverses Hyporesponsiveness and Leads to Filarial Parasite Clearance In Vivo.* J Immunol, 2005. **174**(8): p. 4924-4933.

124. Taylor, M.D., et al., *CTLA-4 and CD4+CD25+ Regulatory T Cells Inhibit Protective Immunity to Filarial Parasites In Vivo.* J Immunol, 2007. **179**(7): p. 4626-4634.

125. Kaplan, M.H., et al., *Th2 Cells Are Required for the Schistosoma mansoni Egg-Induced Granulomatous Response.* J Immunol, 1998. **160**(4): p. 1850-1856.

126. Hoffmann, K.F., A.W. Cheever, and T.A. Wynn, *IL-10 and the Dangers of Immune Polarization: Excessive Type 1 and Type 2 Cytokine Responses Induce Distinct Forms of Lethal Immunopathology in Murine Schistosomiasis.* J Immunol, 2000. **164**(12): p. 6406-6416.

127. Korten, S., et al., *Mast cells in onchocercomas from patients with hyperreactive onchocerciasis (sowda).* Acta Tropica, 1998. **70**(2): p. 217-231.

128. Arndts, K., et al., *Elevated adaptive immune responses are associated with latent infections of Wuchereria bancrofti.* PLoS Negl Trop Dis, 2012. **6**(4): p. e1611.

129. Satoguina, J.S., et al., *Tr1 and naturally occurring regulatory T cells induce IgG4 in B cells through GITR/GITR-L interaction, IL-10 and TGF-β.* Eur J Immunol, 2008. **38**(11): p. 3101-3113.

130. Adjobimey, T., et al., *Co-activation through TLR4 and TLR9 but not TLR2 skews Treg-mediated modulation of Igs and induces IL-17 secretion in Treg: B cell co-cultures.* Innate Immun, 2014. **20**(1): p. 12-23.

131. Yosef, N., et al., *Dynamic regulatory network controlling TH17 cell differentiation.* Nat, 2013. **496**(7446): p. 461-468.

132. Stockinger, B. and M. Veldhoen, *Differentiation and function of Th17 T cells.* Current Opin Immunol, 2007. **19**(3): p. 281-286.

133. Ivanov, I.I., L. Zhou, and D.R. Littman, *Transcriptional regulation of Th17 cell differentiation.* Sem Immunol, 2007. **19**(6): p. 409-417.

134. Ma, C.S., S.G. Tangye, and E.K. Deenick, *Human Th9 cells: inflammatory cytokines modulate IL-9 production through the induction of IL-21.* Immunol Cell Biol, 2010. **88**(6): p. 621-623.

135. Bettelli, E., et al., *Induction and effector functions of TH17 cells.* Nat, 2008. **453**(7198): p. 1051-1057.

136. Zhou, L., M.M.W. Chong, and D.R. Littman, *Plasticity of CD4+ T Cell Lineage Differentiation.* Immun, 2009. **30**(5): p. 646-655.

137. Littman, D.R. and A.Y. Rudensky, *Th17 and Regulatory T Cells in Mediating and Restraining Inflammation.* Cell, 2010. **140**(6): p. 845-858.

138. Lee, Y.K., et al., *Developmental plasticity of Th17 and Treg cells.* Curr Opin Immunol, 2009. **21**(3): p. 274-80.

139. Brattig, N.W., et al., *Immunohistological studies on neoplasms of female and male Onchocerca volvulus: filarial origin and absence of Wolbachia from tumor cells.* Parasitol, 2010. **137**(5): p. 841-54.

140. Rubio de Krömer, M.T., et al., *Detection of a chemotactic factor for neutrophils in extracts of female Onchocerca volvulus.* Acta Tropica, 1998. **71**(1): p. 45-56.

141. Korten, S., et al., *Expansion of NK cells with reduction of their inhibitory Ly-49A, Ly-49C, and Ly-49G2 receptor-expressing subsets in a murine helminth infection: contribution to parasite control.* J Immunol, 2002. **168**(10): p. 5199-206.

142. Babu, S., C.P. Blauvelt, and T.B. Nutman, *Filarial parasites induce NK cell activation, type 1 and type 2 cytokine secretion, and subsequent apoptotic cell death.* J Immunol, 2007. **179**(4): p. 2445-56.

143. Metenou, S., et al., *Filarial Infection Suppresses Malaria-Specific Multifunctional Th1 and Th17 Responses in Malaria and Filarial Coinfections.* J Immunol, 2011. **186**(8): p. 4725-4733.

144. Doetze, A., et al., *Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift.* Int Immunol, 2000. **12**(5): p. 623-30.

145. Korten, S., et al., *Transforming growth factor-beta expression by host cells is elicited locally by the filarial nematode Onchocerca volvulus in hyporeactive patients independently from Wolbachia.* Microbes Infect, 2010. **12**(7): p. 555-64.

146. Wammes, L.J., et al., *Regulatory T Cells in Human Lymphatic Filariasis: Stronger Functional Activity in Microfilaremics.* PLoS Negl Trop Dis, 2012. **6**(5): p. e1655.

147. Taylor, M.D., et al., *Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection.* Eur J Immunol, 2009. **39**(1): p. 192-206.

148. Magg, T., et al., *Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells.* Eur J Immunol, 2012. **42**(6): p. 1627-38.

149. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**(1): p. 485-517.

150. Wang, C., et al., *The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut.* Mucosal Immunol, 2009. **2**(2): p. 173-83.

151. Rottenberg, M.E. and B. Carow, *SOCS3, a major regulator of infection and inflammation.* Front Immunol, 2014. **5**.

152. Bayer, A.L., et al., *A Function for IL-7R for CD4+CD25+Foxp3+ T Regulatory Cells.* J Immunol, 2008. **181**(1): p. 225-234.

153. Hoelzinger, D.B., et al., *Blockade of CCL1 inhibits T regulatory cell suppressive function enhancing tumor immunity without affecting T effector responses.* J Immunol, 2010. **184**(12): p. 6833-42.

154. Hoerauf, A., et al., *The variant Arg110Gln of human IL-13 is associated with an immunologically hyper-reactive form of onchocerciasis (sowda).* Microbes Infect, 2002. **4**(1): p. 37-42.

155. Kerepesi, L.A., et al., *Protective Immunity to the Larval Stages of Onchocerca volvulus Is Dependent on Toll-Like Receptor 4.* Infect Immun, 2005. **73**(12): p. 8291-8297.

156. Brattig, N.W., et al., *The major surface protein of Wolbachia endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4.* J Immunol, 2004. **173**(1): p. 437-45.

157. Lüder, C.G.K., et al., *Immunoregulation in onchocerciasis: predominance of Th1-type responsiveness to low molecular weight antigens of Onchocerca volvulus in exposed individuals without microfilaridermia and clinical disease.* Clin Exp Immunol, 1996. **105**(2): p. 245-253.

158. Mahanty, S., et al., *High levels of spontaneous and parasite antigen-driven interleukin-10 production are associated with antigen-specific hyporesponsiveness in human lymphatic filariasis.* J Infect Dis, 1996. **173**(3): p. 769-73.

159. Elyaman, W., et al., *IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 12885-90.

160. Goswami, R. and M.H. Kaplan, *A brief history of IL-9.* J Immunol, 2011. **186**(6): p. 3283-8.

161. Kaplan, M.H., *Th9 cells: differentiation and disease.* Immunol Rev, 2013. **252**(1): p. 104-115.

162. Beriou, G., et al., *TGF-beta induces IL-9 production from human Th17 cells.* J Immunol, 2010. **185**(1): p. 46-54.

163. Stephens, G.L., et al., *IL-9 is a Th17-derived cytokine that limits pathogenic activity in organ-specific autoimmune disease.* Eur J Immunol, 2011. **41**(4): p. 952-962.

164. Harris, N. and W.C. Gause, *To B or not to B: B cells and the Th2-type immune response to helminths.* Trends Immunol, 2011. **32**(2): p. 80-8.

165. Beagley, K.W., et al., *Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells.* J Exp Med, 1989. **169**(6): p. 2133-2148.

166. Muraguchi, A., et al., *The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells.* J Exp Med, 1988. **167**(2): p. 332-344.

167. Shibui, A., et al., *Th17 cell-derived IL-17 is dispensable for B cell antibody production.* Cytokine, 2012. **59**(1): p. 108-14.

168. Boyer, A.E., et al., *Guatemalan human onchocerciasis. II. Evidence for IgG3 involvement in acquired immunity to Onchocerca volvulus and identification of possible immune-associated antigens.* J Immunol, 1991. **146**(11): p. 4001-10.

169. Mpagi, J.L., et al., *Use of the recombinant Onchocerca volvulus protein Ov20/OvS1 for the immunodiagnostic differentiation between onchocerciasis and mansonelliasis and for the characterization of hyperreactive onchocerciasis (sowda).* Trop Med Int Health, 2000. **5**(12): p. 891-7.

170. Williams, J.F., et al., *Onchocerciasis in Sudan: the Abu Hamed focus.* Trans R Soc Trop Med Hyg, 1985. **79**(4): p. 464-8.

171. Hewitson, J.P., J.R. Grainger, and R.M. Maizels, *Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity.* Mol Biochem Parasitol, 2009. **167**(1): p. 1-11.

172. Marshall, F.A., et al., *ES-62, an Immunomodulator Secreted by Filarial Nematodes, Suppresses Clonal Expansion and Modifies Effector Function of Heterologous Antigen-Specific T Cells In Vivo.* J Immunol, 2005. **175**(9): p. 5817-5826.

173. Grainger, J.R., et al., *Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway.* J Exp Med, 2010. **207**(11): p. 2331-2341.

174. de Jong, E., T. Suddason, and G.M. Lord, *Translational mini-review series on Th17 cells: development of mouse and human T helper 17 cells.* Clin Exp Immunol, 2010. **159**(2): p. 148-58.

175. He, R., et al., *Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13.* J Allergy Clin Immunol, 2009. **124**(4): p. 761-70.

176. Soboslay, P.T., et al., *Prenatal immune priming in onchocerciasis-onchocerca volvulus-specific cellular responsiveness and cytokine production in newborns from infected mothers.* Clin Exp Immunol, 1999. **117**(1): p. 130-7.

177. Bourke, C.D., R.M. Maizels, and F. Mutapi, *Acquired immune heterogeneity and its sources in human helminth infection.* Parasitol, 2011. **138**(2): p. 139-59.

178. Debrah, A.Y., et al., *Plasma Vascular Endothelial Growth Factor-A (VEGF-A) and VEGF-A Gene Polymorphism are Associated with Hydrocele Development in Lymphatic Filariasis.* Am J Trop Med Hyg, 2007. **77**(4): p. 601-608.

179. Ali, M.M., et al., *Fc gamma RIIa (CD32) polymorphism and onchocercal skin disease: implications for the development of severe reactive onchodermatitis (ROD).* Am J Trop Med Hyg, 2007. **77**(6): p. 1074-8.

180. Pfaff, A.W., et al., *Regulatory effects of IL-12 and IL-18 on Onchocerca volvulus- and Entamoeba histolytica-specific cellular reactivity and cytokine profiles.* Parasite Immunol, 2003. **25**(6): p. 325-32.

6. APPENDIX

Appendix A: EQUIPMENTS

Equipments	Company, city and country
BD FACS Canto™ flow cytometer	BD Biosciences, Heidelberg, Germany
Cellstar 96-Well Cell Culture Plate, sterile, U-bottom, with lid	Greiner bio-one, Frickenhausen, Germany
Cellstar serological pipette, 5mL, 10mL	Greiner bio-one, Frickenhausen, Germany
Cellstar Tubes (15ml, 50ml)	Greiner bio-one, Frickenhausen, Germany
Centrifuge	Kendro, Langenselbold, Germany
Costar Stripette, serological pipette, 25mL	Corning Incorporated, Corning, New York, USA
Cryo Tubes	Thermo Scientific, Roskilde, Denmark
Falcon serological pipette, 50 mL	Becton Dickinson Labware, Franklin Lakes, NJ. USA
Freezer (-20°C)	AEG, Nürnberg, Germany

Freezer (-80°C)	AEG, Nürnberg, Germany
Fridge	Liebherr, Biberach an der Riss, Germany
Glass Pasteur Pipette	Brand GmbH + CO, Wertheim, Germany
Incubator	Kendro, Langenselbold, Germany
Lamina Flow hood	Kendro, Langenselbold, Germany
MACS multistand separator	Miltenyi BiotecGmbH, Bergisch Gladbach, Germany
MACS separation columns	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Micropipette Eppendorf Research	Eppendorf AG, Hamburg, Germany
Microscope	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
Primus Thermocycler	MWG-Biotech, Ebersberg, Germany
Multichannel pipette (m300)	Biohit, Göttingen, Germany
Multipette stream	Eppendorf AG, Hamburg, Germany
Neubauer counting chamber	Marienfeld, Lauda Königshofen, Germany
Pipetboyacu	Integra Biosciences, Fernwald, Germany
PS Microplates	Greiner bio-one, Frickenhausen, Germany

Plate reader SpectraMAX 340 Pc	Molecular Devices
NanoDrop 1000	Peqlab, Erlangen, Germany
Round-Bottom Tubes (5mL)	Becton Dickinson Labware, Franklin Lakes, NJ. USA
Safe-Lock Tubes (0.5-2ml)	Eppendorf AG, Hamburg, Germany
Tip One, pipette tips	Starlab Group, Hamburg, Germany
Vacuum pump	ABM Greiffenberger, Marktredwitz, Germany
Vortex mixer (Minishaker)	VWR International, Darmstadt, Germany
Water bath	VWR International, Darmstadt, Germany

Appendix B: Chemicals and Reagents

Reagents	Company, city and country
Advanced RPMI 1640 (1x)	Life Technologies Corporation, Grand Island, NY, USA
Anti-Human CD25 FITC	Ebioscience, San Diego, USA
Anti-Human CD25 PE-Cy7	Ebioscience, San Diego, USA
Anti-Human CD4 APC	Ebioscience, San Diego, USA
Anti-Human CTLA-4 PE	Ebioscience, San Diego, USA
Anti-Human Foxp3 PE	Ebioscience, San Diego, USA
Anti-Human IFN_Y PE	Ebioscience, San Diego, USA
Anti-Human IL-10	Immuno Tools, Friesoythe, Germany
Anti-human IL-13	Immuno Tools, Friesoythe, Germany
Anti-Human IL-17A PE	Ebioscience, San Diego, USA
Anti-Human IL-4	Immuno Tools, Friesoythe, Germany
Anti-Human IL-4 PE	Ebioscience, San Diego, USA
C12-iE-DAP, Human NOD1 ligand	InvivoGen, San Doiego, USA
CD14 MicroBeads, human	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

CD19 MicroBeads, human	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD3/CD28 MACSiBead Particles	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD4 MicroBeads, human	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD4+CD25+CD127dim/- Reg. T Cell Isolation Kit II	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Cell stimulation Cocktail plus golgiStop	Ebioscience, San Diego, USA
Coomassie blue G	Cytoscelecton, Denver, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
DNA-free	
Dulbecco's PBS	Life Technologies Corporation, Paisley, UK
Dynabeads Human T-Activator CD3/CD28	Invitrogen Dynal, Oslo, Norway
Fetal Calf Serum, Standard Quality	PAA Laboratories GmbH, Pasching, Austria
Fixation/Permeabilization Concentrate	ebioscience, San Diego, USA

Fixation/Permeabilization Diluent	ebioscience, San Diego, USA
Gentamycin (50 mg mL^{-1})	PAA Laboratories GmbH, Pasching, Austria
HKLM, Human TLR2 ligand	InvivoGen, San Doiego, USA
Human 13-plex cytokines Kit	Ebioscience, San Diego, USA
Human Immunoglobulin Isotyping Panel 6-plex Kit	Ebioscience, San Diego, USA
Human Immunoglobulin simplex IgE Kit	Ebioscience, San Diego, USA
L-Glutamine 200 mM (100x)	Life Technologies Corporation, Grand Island, NY, USA
LPS-EK, Human TLR4 ligand	InvivoGen, San Doiego, USA
MACS Separation Buffer	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Master mix	Quiagen GmbH, Hilden, Germany
MDP, Human NOD2 ligand	InvivoGen, San Doiego, USA
Normal Rat Serum	Ebioscience, San Diego, USA
ODN 2006 Human TLR9 ligand	InvivoGen, San Doiego, USA
Pam3CSK, Human TLR1/2 ligand	InvivoGen, San Doiego, USA

Pancoll Human (1,077 g mL^{-1})	PAN Biotech, Aidenbach, Germany
Penicillin Streptomycin	Life Technologies Corporation, Grand Island, NY, USA
Permeabilization Buffer (10x)	Ebioscience, San Diego, USA
Recombinant Human Interleukin-10	Immuno Tools, Friesoythe, Germany
Recombinant Human Interleukin-13	Immuno Tools, Friesoythe, Germany
Recombinant Human Interleukin-1β	Immuno Tools, Friesoythe, Germany
Recombinant Human Interleukin-4	Immuno Tools, Friesoythe, Germany
Recombinant Human Interleukin-6	Immuno Tools, Friesoythe, Germany
Trizol	Invitrogen
Trypan blue 2 % in PBS	
Ultra Comp ebeads	Ebioscience, San Diego, USA

Appendix C: FACS staining antibodies

Antibodies	**Clone**	**Company, city and country**
Anti-Human CD14-FITC	61D3	Ebioscience, San Diego, USA
Anti-Human CD16-FITC	CB16	Ebioscience, San Diego, USA
Anti-Human CD19-APC	HIB19	Ebioscience, San Diego, USA
Anti-Human CD25-PE-Cy7	BC96	Ebioscience, San Diego, USA
Anti-Human CD27-FITC		Ebioscience, San Diego, USA
Anti-Human CD3-PerCP Cy5.5	OKT3	Ebioscience, San Diego, USA
Anti-Human CD45RA-PerCPCy5.5	HI100	Ebioscience, San Diego, USA
Anti-Human CD45RO-FITC	UCHL	Ebioscience, San Diego, USA
Anti-Human CD4-APC	OKT4	Ebioscience, San Diego, USA
Anti-Human CD56-PE	CMSSB	Ebioscience, San Diego, USA
Anti-Human CD8-APC	SK1	Ebioscience, San Diego, USA

Anti-Human Foxp3 PE	236A/E7	Ebioscience, San Diego, USA
Anti-Human GATA3-PE	JWAJ	Immuno Tools, Friesoythe, Germany
Anti-Human IFN$_{\gamma}$ PE	4S.B3	Ebioscience, San Diego, USA
Anti-Human IL-10-PE	JES3-9D7	Immuno Tools, Friesoythe, Germany
Anti-Human IL-17A-FITC	AFKJS-9	Ebioscience, San Diego, USA
Anti-Human IL-4-PE	B-S4	Ebioscience, San Diego, USA
Anti-Human RORC2-PE	AFKJS-9	Ebioscience, San Diego, USA
Anti-Human T bet-PE	eBio64DE17	Ebioscience, San Diego, USA

Appendix D: Software

BD FACSDiver software, BD Biosciences, Heidelberg, Germany

SoftMax Pro 5.0 software, Molecular Devices, Ismning, Germany

RT^2 profiler PCR Array data analysis 3.5 software, Qiagen, Hilden, Germany

PRISM 5.02, GraphPad Software, Inc., La Jolla, USA

Appendix E: Buffers and Solutions

Cell culture medium

500ml RPMI 1640 medium

5ml Penicillin (100µg/ml)/Streptomycin (100µg/ml)

0.5 ml Gentamycin (50µg/ml)

5ml L-glutamine (292.3µg/ml)

10% FCS

For 50ml: 5ml FCS + 45ml RPMI 1640 culture medium with antibiotics

Coating buffer (ELISA)

0.1M of $NaHCO_3$

For 1 liter: 8.4g $NaHCO_3$ in 900ml distilled water, adjust the PH to 9.6 using KOH, then top to 1 liter

Blocking buffer (ELISA)

Assay diluent (5x), dilution 1:5

For 200ml; 40ml assay diluents 5x + 160ml distilled water

FACS Buffer

1xPBS with 2 % FCS

PFA 4% solution (FACS)

40g of PFA dissolved while heating (max 60°C) in 900ml 1x PBS. Increase solubility by adding NaOH dropwise, top to 1 liter with 1x PBS. Solution then cooled and filtered. Adjust pH to 6.9 using dilute HCl.

Fixation/Permeabilisation Buffer

1:4 dilution

For 12ml: 3ml Fix/perm concentrate + 9ml dilution buffer

Permeabilization Buffer

1:10 dilution

For 50ml: 5 ml 10x permeabilisation buffer + 45ml distilled water

Stop Solution 2N H_2SO_4

½ Liter: 26,6ml H_2SO_4 +473ml distilled water

Wash buffer

For 5 liters:

weigh out 47.75g of PBS

Fill up with distilled water

2.5ml Tween 80

adjust pH 7.2-7.4

Appendix F: PCR Array. Gene table

Position	Symbol	Description	Gene name
A01	CCL1	Chemokine (C-C motif) ligand 1	I-309/P500/SCYA1/SISe/TCA3
A02	CCL2	Chemokine (C-C motif) ligand 2	GDCF-2/HC11/HSMCR30/MCAF/MCP-1/MCP1/SCYA2/SMC-CF
A03	CCL20	Chemokine (C-C motif) ligand 20	CKb4/LARC/MIP-3-alpha/MIP-3a/MIP3A/SCYA20/ST38
A04	CCL22	Chemokine (C-C motif) ligand 22	ABCD-1/DC/B-CK/MDC/SCYA22/STCP-1
A05	CCL7	Chemokine (C-C motif) ligand 7	FIC/MARC/MCP-3/MCP3/NC28/SCYA6/SCYA7
A06	CCR2	Chemokine (C-C motif) receptor 2	CC-CKR-2/CCR-2/CCR2A/CCR2B/CD192/CKR2/CKR2A/CKR2B/CMKBR2/MCP-1-R
A07	CCR4	Chemokine (C-C motif) receptor 4	CC-CKR-4/CD194/CKR4/CMKBR4/ChemR13/HGCN:14099/K5-5
A08	CCR6	Chemokine (C-C motif) receptor 6	BN-1/C-C CKR-6/CC-CKR-6/CCR-6/CD196/CKR-L3/CKRL3/CMKBR6/DCR2/DRY6/Y4/STRL22
A09	CD28	CD28 molecule	Tp44
A10	CD34	CD34 molecule	-
A11	CD4	CD4 molecule	CD4mut
A12	CD40LG	CD40 ligand	CD154/CD40L/HIGM1/IGM/IMD3/T-BAM/TNFSF5/TRAP/gp39/hCD40L
B01	CD8A	CD8a molecule	CD8/Leu2/MAL/p32
B02	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	C/EBP-beta/CRP2/IL6DBP/LAP/LIP/NF-IL6/TCF5

B03	CLEC7A	C-type lectin domain family 7, member A	BGR/CANDF4/CLECSF12/DECTIN1
B04	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	GMCSF
B05	CSF3	Colony stimulating factor 3 (granulocyte)	C17orf33/CSF3OS/GCSF
B06	CX3CL1	Chemokine (C-X3-C motif) ligand 1	ABCD-3/C3Xkine/CXC3/CXC3C/NTN/NTT/SCYD1/fractalkine/neurotactin
B07	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	FSP/GRO1/GROa/MGSA/MGSA-a/NAP-3/SCYB1
B08	CXCL12	Chemokine (C-X-C motif) ligand 12	IRH/PBSF/SCYB12/SDF1/TLSF/TPAR1
B09	CXCL2	Chemokine (C-X-C motif) ligand 2	CINC-2a/GRO2/GROb/MGSA-b/MIP-2a/MIP2/MIP2A/SCYB2
B10	CXCL5	Chemokine (C-X-C motif) ligand 5	ENA-78/SCYB5
B11	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CKA-3/GCP-2/GCP2/SCYB6
B12	FOXP3	Forkhead box P3	AIID/DIETER/IPEX/PIDX/XPID
C01	GATA3	GATA binding protein 3	HDR/HDRS
C02	ICAM1	Intercellular adhesion molecule 1	BB2/CD54/P3.58
C03	ICOS	Inducible T-cell co-stimulator	AILIM/CD278/CVID1
C04	IFNG	Interferon, gamma	IFG/IFI
C05	IL10	Interleukin 10	CSIF/GVHDS/IL-10/IL10A/TGIF
C06	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	CLMF/CLMF2/IL-12B/NKSF/NKSF2

C07	IL12RB1	Interleukin 12 receptor, beta 1	CD212/IL-12R-BETA1/IL12RB
C08	IL12RB2	Interleukin 12 receptor, beta 2	-
C09	IL13	Interleukin 13	IL-13/P600
C10	IL15	Interleukin 15	IL-15
C11	IL17A	Interleukin 17A	CTLA8/IL-17/IL-17A/IL17
C12	IL17C	Interleukin 17C	CX2/IL-17C
D01	IL17D	Interleukin 17D	IL-17D/IL-27/IL27
D02	IL17F	Interleukin 17F	CANDF6/IL-17F/ML-1/ML1
D03	IL17RA	Interleukin 17 receptor A	CANDF5/CD217/CDw217/IL-17RA/IL17R/hIL-17R
D04	IL17RB	Interleukin 17 receptor B	CRL4/EVI27/IL17BR/IL17RH1
D05	IL17RC	Interleukin 17 receptor C	IL17-RL/IL17RL
D06	IL17RE	Interleukin 17 receptor E	-
D07	IL18	Interleukin 18 (interferon-gamma-inducing factor)	IGIF/IL-18/IL-1g/IL1F4
D08	IL1B	Interleukin 1, beta	IL-1/IL1-BETA/IL1F2
D09	IL1R1	Interleukin 1 receptor, type I	CD121A/D2S1473/IL-1R-alpha/IL1R/IL1RA/P80
D10	IL2	Interleukin 2	IL-2/TCGF/lymphokine

D11	IL21	Interleukin 21	IL-21/Za11
D12	IL22	Interleukin 22	IL-21/IL-22/IL-D110/IL-TIF/ILTIF/TIFIL-23/TIFa/zcyto18
E01	IL23A	Interleukin 23, alpha subunit p19	IL-23/IL-23A/IL23P19/P19/SGRF
E02	IL23R	Interleukin 23 receptor	-
E03	IL25	Interleukin 25	IL17E
E04	IL27	Interleukin 27	IL-27/IL-27A/IL27A/IL27p28/IL30/p28
E05	IL3	Interleukin 3 (colony-stimulating factor, multiple)	IL-3/MCGF/MULTI-CSF
E06	IL4	Interleukin 4	BCGF-1/BCGF1/BSF-1/BSF1/IL-4
E07	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	EDF/IL-5/TRF
E08	IL6	Interleukin 6 (interferon, beta 2)	BSF2/HGF/HSF/IFNB2/IL-6
E09	IL6R	Interleukin 6 receptor	CD126/IL-6R-1/IL-6RA/IL6Q/IL6RA/IL6RQ/gp80
E10	IL7R	Interleukin 7 receptor	CD127/CDW127/IL-7R-alpha/IL7RA/ILRA
E11	IL8	Interleukin 8	CXCL8/GCP-1/GCP1/LECT/LUCT/LYNAP/MDNCF/MONAP/NAF/NAP-1/NAP1
E12	IL9	Interleukin 9	HP40/IL-9/P40
F01	IRF4	Interferon regulatory factor 4	LSIRF/MUM1/NF-EM5
F02	ISG20	Interferon stimulated exonuclease gene 20kDa	CD25/HEM45

F03	JAK1	Janus kinase 1	JAK1A/JAK1B/JTK3
F04	JAK2	Janus kinase 2	JTK10/THCYT3
F05	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	CHDS6/MMP-3/SL-1/STMY/STMY1/STR1
F06	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	CLG4B/GELB/MANDP2/MMP-9
F07	NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	NFAT1/NFATP
F08	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1/KBF1/NF-kB1/NF-kappa-B/NF-kappaB/NFKB-p105/NFKB-p50/NFkappaB/p105/p50
F09	RORA	RAR-related orphan receptor A	NR1F1/ROR1/ROR2/ROR3/RZR-ALPHA/RZRA
F10	RORC	RAR-related orphan receptor C	NR1F3/RORG/RZR-GAMMA/RZRG/TOR
F11	RUNX1	Runt-related transcription factor 1	AML1/AML1-EVI-1/AMLCR1/CBFA2/EVI-1/PEBP2aB
F12	S1PR1	Sphingosine-1-phosphate receptor 1	CD363/CHEDG1/D1S3362/ECGF1/EDG-1/EDG1/S1P1
G01	SOCS1	Suppressor of cytokine signaling 1	CIS1/CISH1/JAB/SOCS-1/SSI-1/SSI1/TIP3
G02	SOCS3	Suppressor of cytokine signaling 3	ATOD4/CIS3/Cish3/SOCS-3/SSI-3/SSI3
G03	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	APRF/HIES
G04	STAT4	Signal transducer and activator of transcription 4	SLEB11
G05	STAT5A	Signal transducer and activator of transcription 5A	MGF/STAT5
G06	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	D12S1644/IL-4-STAT/STAT6B/STAT6C

G07	SYK	Spleen tyrosine kinase	p72-Syk
G08	TBX21	T-box 21	T-PET/T-bet/TBET/TBLYM
G09	TGFB1	Transforming growth factor, beta 1	CED/DPD1/LAP/TGFB/TGFbeta
G10	TLR4	Toll-like receptor 4	ARMD10/CD284/TLR-4/TOLL
G11	TNF	Tumor necrosis factor	DIF/TNF-alpha/TNFA/TNFSF2
G12	TRAF6	TNF receptor-associated factor 6	MGC:3310/RNF85
H01	ACTB	Actin, beta	BRWS1/PS1TP5BP1
H02	B2M	Beta-2-microglobulin	-
H03	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
H04	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
H05	RPLP0	Ribosomal protein, large, P0	L10E/LP0/P0/PRLP0/RPP0
H06	HGDC	Human Genomic DNA Contamination	HIGX1A
H07	RTC	Reverse Transcription Control	RTC
H08	RTC	Reverse Transcription Control	RTC
H09	RTC	Reverse Transcription Control	RTC
H10	PPC	Positive PCR Control	PPC

H11	PPC	Positive PCR Control	PPC
H12	PPC	Positive PCR Control	PPC

ERKLÄRUNG

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbst und ohne jede unerlaubte Hilfe angefertigt habe, daß diese oder eine ähnliche Arbeit noch keiner anderen Stelle als Dissertation eingereicht worden ist.

Bonn, den.....

Gnatoulma Katawa

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